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(57) Abstract

This invention relates to nucleic acid, or fragments thereof, encoding the retinoblastoma polypeptide, the retinoblastoma polypeptide itself, methods of detecting a defective retinoblastoma gene in human patients, and methods of treating these patients.

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DIAGNOSIS OF RETINOBLASTOMA

Background of the Invention

This application is a continuation-in-part of Dryja et al. U.S.S.N. 146,525, filed January 21, 1988, which is a continuation-in-part of Dryja et al. U.S.S.N. 895,163, filed August 11, 1986, both hereby incorporated by reference.

This invention concerns the retinoblastoma gene and methods for detecting and treating patients afflicted with a defective retinoblastoma gene.

Retinoblastoma is a neoplastic condition of the retinal cells, observed almost exclusively in children between the ages of 0 and 4 years. It affects between 1 in 34,000 and 1 in 15,000 live births in the United States. (L.E. Zimmerman, 1985, Retinoblastoma and retinocytoma, In W.H. Spencer (ed.), Ophthalmic Pathology: an Atlas and Textbook, Vol. II, Philadelphia: W.B. Saunders Co., pp. 1292-1351.) If untreated, the malignant neoplastic retinal cells in the intraocular tumor travel to other parts of the body, forming foci of uncontrolled growth which are always fatal. The current treatment for a retinoblastoma is enucleation of the affected eye if the intraocular tumor is large; for small intraocular tumors, radiation therapy, laser therapy, or cryotherapy is preferred. There is no known successful treatment for metastatic retinoblastoma. As with most cancers, morbidity and mortality are reduced if diagnosis can be made early in the course of the disease.

In 30-40% of cases of retinoblastoma, the affected individual carries a heritable predisposition to retinoblastoma and can transmit this predisposition to his or her offspring as a dominant trait (A.G. Knudson, 1971, Mutation and cancer: Statistical study of retinoblastoma, Proc. Natl. Acad. Sci., Vol. 68, pp. 820-23). Carriers of this retinoblastoma-predisposing trait are at a greatly elevated risk for development of several other forms of primary cancer, notably osteosarcoma and soft-tissue sarcoma.

The genetic locus associated with familial retinoblastoma has been assigned to the q14 band of human chromosome 13 (R.S. Sparkes et al., 1980, Science, Vol. 208, pp. 1042-44). Most retinoblastomas arise from cells which have lost both normal, dominant, homologous alleles at this retinoblastoma locus. However, individuals carrying one defective allele may be predisposed to the disease. Children who have had one eye affected by retinoblastoma or who are related to someone with retinoblastoma may be genetically predisposed and therefore at risk of developing the disease. These individuals routinely are tested for retinoblastoma every 2-3 months by an ocular examination procedure which requires placing the child under general anesthesia.

Summary of the Invention

In general, the invention concerns purified nucleic acid (less than 100kb in size), and fragments thereof of at

least 15 bases, encoding the Rb gene. The invention also concerns cells transformed with this nucleic acid, isolated polypeptides encoded by this nucleic acid, and antibodies to this polypeptide, or to naturally occurring retinoblastoma polypeptide. Retinoblastoma polypeptide is the polypeptide encoded by the Rb gene. Further, the invention concerns a composition, suitable for treating a human having a defective Rb gene, containing retinoblastoma polypeptide, or a fragment thereof, in a pharmacologically acceptable carrier.

The invention also features methods of screening human patients to determine those not at risk of developing retinoblastoma and thus not requiring conventional examinations to be performed. This screening involves, for example, comparing nucleic acid of a patient with purified nucleic acid encoding a human Rb gene, or fragments thereof.

Thus in various aspects, the invention features methods of analyzing the predisposition of patients to retinoblastoma which involves detecting large and small deletions or point mutations in the retinoblastoma gene, or detecting the co-inheritance of such defects with specific restriction fragment length polymorphisms (RFLPs), or detecting the presence or absence of a normal or defective retinoblastoma gene by hybridizing a nucleic acid sample from the patient with a probe specific for the retinoblastoma gene, and determining the ability of the probe to hybridize to the nucleic acid. The

lack of hybridization to the nucleic acid indicates the presence of a large deletion in the gene. A probe specific for the retinoblastoma gene may be hybridized to fragments separated by a defined physical property from a sample of a patient, the hybrids of the probe and the fragments detected, and the hybrids compared to hybrids detected from the hybridization of the probe and separated nucleic acid fragments from a normal retinoblastoma gene. The absence of hybrids or presence of hybrids of a smaller size compared to a normal patient is an indication of large deletions in the retinoblastoma gene of the patient. Preferably, the probe specific for the retinoblastoma gene is the cloned DNA in p4.7R; or a fragment thereof; and the defined physical property is molecular weight.

Small deletions or point mutations can be detected by determining the nucleotide sequence of a retinoblastoma allele from a patient, and comparing the nucleotide sequence with the nucleotide sequence of a retinoblastoma allele, or subregion thereof, from a person not afflicted with retinoblastoma; or by detecting mismatches between a nucleic acid sample from a patient and a probe specific for the retinoblastoma gene from a person not afflicted with retinoblastoma. The co-inheritance of specific genetic polymorphisms with the retinoblastoma gene may be an indication of the predisposition of a patient to retinoblastoma. According to this method, nucleic acid

fragments are generated from a sample of the patient, the fragments are separated according to a defined physical property of the fragments (e.g., molecular weight), a detectable probe specific for the retinoblastoma gene is hybridized to the fragments, hybrids of the probe and the fragments are detected, and the hybrids are compared to hybrids detected from the hybridization of the same probe and separated nucleic acid fragments from a sample of a parent of the patient.

In another aspect, the invention features the use of an isolated normal human retinoblastoma gene to synthesize Rb polypeptide for use in the treatment of individuals determined to have a defective Rb allele.

In yet another aspect, the invention features a method of detecting the presence of the retinoblastoma polypeptide in a tumor sample from a human patient, by producing an antibody to the retinoblastoma polypeptide, contacting the antibody with the tumor sample, and detecting immune complexes as an indication of the presence in the tumor sample of the retinoblastoma polypeptide. The absence of the polypeptide indicates that the tumor is caused by a defect in a retinoblastoma allele. This procedure would preferably involve contacting a tumor sample from a human patient with an antibody (e.g., monoclonal antibody) which specifically reacts with the retinoblastoma polypeptide, or a fragment thereof, and determining whether the antibody binds to cells of the tissue

specimens. The absence of immune complexes is an indication that the tumor was the result of a defective retinoblastoma allele.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The description of the preferred embodiments of the invention will follow the brief description of the drawings given below.

Drawings

Fig. 1 is a diagrammatic representation of a restriction map of the insert in the clone p4.7R.

Fig. 2 is a diagrammatic representation of a restriction map of the genomic locus of the retinoblastoma gene.

Fig. 3 is a diagrammatic representation of the vectors p2AR3.8 and p2AR0.9 of the invention.

Fig. 4 is a scale map of the normal retinoblastoma gene.

Fig. 5 (5-1 through 5-3) is a nucleic acid sequence of a cDNA of the normal retinoblastoma gene, with flanking regions.

Fig. 6 (6-1 through 6-9) is a nucleic acid sequence of exons of the normal retinoblastoma gene, with flanking regions.

Fig. 7 is a restriction map of the retinoblastoma gene, showing the locations of DSPs.

Fig. 8 is a gel and a diagram showing the inheritance og the polymorphism RB1.3 in a retinoblastoma-prone family.

Fig. 9 is a diagram showing the segregation of the DSP RB1.3 in three families with hereditary retinoblastoma.

Retinoblastoma Polypeptide

The Rb polypeptide is the specific amino acid chain encoded by the nucleic acid sequence of the normal retinoblastoma gene. The Rb polypeptide of this invention includes: (1) naturally occurring retinoblastoma protein; (2) synthetically produced retinoblastoma polypeptide; and (3) retinoblastoma polypeptide produced from purified nucleic acid (e.g., cDNA or genomic DNA) via an in vitro expression system. Also included are biologically active fragments of Rb polypeptide which either have a biological activity of naturally occurring Rb polypeptide, or include an epitope of this polypeptide and thus are suitable for production of Rb-specific antibodies.

Retinoblastoma Gene

The Rb gene is that distinct nucleic acid sequence in the human genome, the absence or mutation of which predisposes one to retinoblastoma. The purified nucleic acid sequence encoding the retinoblastoma gene can be carried on vectors which can be propagated in cells. For the purposes of this invention, purified nucleic acid encoding the Rb gene is defined as nucleic acid isolated from its natural environment

(e.g., cDNA or a fragment of genomic DNA) which hybridizes specifically to the retinoblastoma gene under hybridizing conditions. An example of purified nucleic acid which encodes the retinoblastoma gene, and is carried on a vector, is the cDNA clone p4.7R. This clone was obtained in the following manner.

cDNA

The human DNA probe pH3-8, isolated from a human chromosome 13 lambda phage library (M. Lalande et al., 1984, Cancer Genet. Cytogenet., Vol. 13, pp. 283-95), was used in a chromosome walking technique to isolate and map 30 kilobases (kb) of genomic DNA surrounding the H3-8 sequence. One fragment generated by this technique, named p7H30.7R, was found to recognize a DNA sequence in the mouse genome as well as one within human chromosome 13 (T.P. Dryja et al., 1986, Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 7391-94). The homology of p7H30.7R to both human and mouse DNA suggested that p7H30.7R contains coding sequences of a structural gene.

To test this possibility, p7H30.7R was radiolabeled and used to probe a Northern blot of RNA isolated from three retinoblastoma tumors and an adenovirus 12-transformed human embryonic retinal cell line (Vaessen et al., 1986, EMBO Journal, Vol. 5, pp. 335-). The p7H30.7R probe hybridized to an RNA transcript of approximately 4.7 kb from the retinal cell line, but did not hybridize to any RNA transcripts from the

three tumor samples.

Subsequently, RNA isolated from the adenovirus-transformed retinal cell line was used to construct a cDNA library. This library was screened with the labeled p7H30.7R probe. Several cDNA clones were isolated which had similar restriction maps. The longest of these, p4.7R, contained 4.7 kb of DNA. The restriction map of the insert in the clone p4.7R is shown in Fig. 1.

The p4.7R clone was used to screen RNA transcripts isolated from four retinoblastomas, an osteosarcoma, and the adenovirus-transformed retinal cells. In a Northern blot analysis of isolated RNA's, the p4.7R probe cross-reacted with a 4.7 kb transcript in the transformed retinal cells which was not present in the four retinoblastoma and one osteosarcoma cell samples.

Genomic DNA

Clones containing genomic DNA including the retinoblastoma gene were isolated in the manner described below. Recombinant bacteriophage libraries containing human genomic DNA fragments inserted in the lambda phage cloning vector EMBL-3 were constructed according to published methods (Seed et al., 1982, Gene, Vol. 19, pp. 201-209). Those recombinant bacteriophage which contain fragments of the retinoblastoma gene were initially detected by hybridization of the bacteriophage plaques with p4.7R.

Thirty-six distinct recombinant bacteriophage that contain overlapping human genomic DNA fragments were isolated. Selected bacteriophage were plaque-purified and amplified, and the restriction map of each phage insert was determined by the method of Rackwitz et al., Gene, Vol. 30, pp. 195-200.

With these bacteriophage a restriction map of a region that spans approximately 200 kb was constructed, shown in Figs. 2 and 4. All of the known sequences present in the mRNA from the retinoblastoma gene are present in this cloned region. In aggregate, the human DNA sequences in this set of bacteriophage represent the chromosomal segment of the Rb gene. In Fig. 2, the vertical marks above the map represent the location of HindIII sites, and the vertical marks below the map represent the location of EcoRI sites. The boxed areas represent HindIII fragments which contain sequences found in the cDNA (exons). Each double-headed arrow beneath the map represents a distinct recombinant bacteriophage clone. Fig. 4 shows all the recognition sites of the six restriction enzymes Hind III, EcoR I, Xba I, Sac I, Sac II, and BamH I (New England Biolabs, Inc.). Restriction endonuclease fragments that contained exons were identified by their hybridization with cDNA clones or synthesized oligonucleotide sequences based on cDNA sequence. These restriction fragments were subcloned in the plasmid vector Bluescribe (Stratagene, San Diego, CA). A total of 24 distinct plasmids were subcloned in this manner.

The number and size of each exon was determined by iterations of the following procedure. First, an oligonucleotide was synthesized that corresponded to the first 20 nucleotides of the cDNA sequence. Using this oligonucleotide as a primer, the plasmid with a genomic insert containing the most 5' exon was sequenced. The resultant sequence was aligned with the cDNA sequence to determine the length of the first exon; the point at which the plasmid and cDNA sequences diverged marked the beginning of the first intron. This exon and the flanking regions were further sequenced using synthetic oligonucleotide primers to generate a continuous nucleotide sequence composed of 5' promoter sequence, exon 1, and the beginning of intron 1. The second and subsequent exons were defined by synthesizing sequencing primers corresponding to the next 20 nucleotides of cDNA sequence that had not been previously assigned to an exon. All exons and the immediately adjacent flanking intron sequences were sequenced in both sense and antisense directions.

The dideoxynucleotide chain termination method of sequencing was carried out using the enzyme Sequenase (United States Biochemical Corporation, Cleveland, Ohio) according to protocols supplied by the manufacturer. The intron region downstream of exon 20 could not be sequenced by this method, due to an unusually problematic repeated sequence in this region that caused a series of 45 stops (bands appeared in all

four lanes of the sequencing gel). To resolve this region, sequencing reactions were carried out with Taq polymerase (Perkin-Elmer/Cetus). This enzyme allowed for the polymerization to be performed at 68°C and resolved the bases in this region.

All sequence data were analyzed and screened for overlapping regions using the sequence analysis program Microgenie Sequence Software (Beckman, Palo Alto, CA).

The position of each exon within the restriction map of the gene was determined by hybridization of cDNA fragments or synthetic oligomer sequences to recombinant bacteriophage DNA that had been digested with various restriction endonucleases. The precise location of most exons was subsequently deduced when recognition sequences of endonucleases were identified within the intron-exon sequence and correlated with the map. The position of each of the remaining exons was arbitrarily placed in the center of the smallest restriction fragment to which it hybridized.

The organization of the 27 exons along the genomic map of the retinoblastoma gene is illustrated in figure 4. This figure details the recognition sites for 6 restriction endonucleases and the position of the exons relative to these sites. Exons 1, 2, 3, 6, 9, 10, 13, 21, 22, 23, 24, 235, 26, and 27 have been precisely localized on this map. The other exons were mapped within small restriction fragments and are

illustrated in the middle of these fragments. Exons 11, 17, and the cluster of exons 14-16 were mapped by this technique with uncertainties of not more than 2.0 kb. The remaining exons (exons 4, 5, 7, 8, 12, 18, 19, and 20) were all mapped to within 0.8 kb of their true locations. For reference, this map also shows the position of several naturally occurring restriction fragment length polymorphisms.

Fig. 6 (6-1 through 6-9) shows the sequence flanking and including each exon. The exons range in size from 31 nucleotides (exon 24), to 1973 nucleotides (exon 27). The shortest intron sequence was found to be 80 nucleotides long and is located between exons 15 and 16, whereas the largest spans approximately 70.5 kb between exon 17 and 18. All of the intron donor and acceptor splicing sites comply with the GT-AG splice junction rule. Our methods of sequencing proved more accurate than previous reports in defining the exact number of exons comprising the retinoblastoma gene.

The first exon and the region immediately 5' to this exon are very G-C rich, which is a characteristic of promoter regions. This region contains 9 possible Hpa II restriction sites, is composed of 66% C+G nucleotides and does not exhibit CpG suppression. These criteria are indicative of a HTF island. This promoter region contained some nucleotides that could not be resolved in either the sense or antisense direction using either Sequenase or Taq polymerase.

Presumably, this was due to secondary structure that forms in this promoter region.

Analysis of the sequence approximately 30 nucleotides upstream of the transcription initiation site defined by Lee et al. (1987b) does not reveal a TATA box that is found in other promoter regions. This suggests that either the previously published initiation site is not in fact correctly defined, or that the retinoblastoma gene lacks the prototypical TATA and CAAT boxes of promoter regions. Further analysis of the sequence 5' to exon 1 reveals a possible TATA box at base pair #-274, labeled base pair #122 in figure 6-1. Homology for the seven base region is only 57%, yet the first four bases T-A-T-A, which are the bases most frequently conserved, are 100% homologous. A possible capping site, CAC, is located 14 bases away and again 49 bases away. CAAT boxes were not identified although the region is generally G-C rich.

The intron sequence that flanks the 3' side of exon 20 consisted of 21 consecutive repeats of the sequence TTT(T)C that together span 87 nucleotides. The number of repeat units can vary between different individuals and the alleles determined by the number of repeats behave like a heritable DNA polymorphism.

A computer search of the sequence data identified several intron regions homologous to Alu repetitive sequences. Alu repeats were located in the following regions: (1)

downsteam of exon 2, between bp 492 and bp 704 according to the numbering scheme in Figure 6; 2) upstream of exon 9, between bp 19 and bp 117; 3) downstream of exon 11, between bp 504 and bp 680; 4) the intron sequnce flanking both sides of exon 14 between bp 132 and bp 270 and between bp 420 and bp 741; and, 5) upstream of exon 17, between bp 36 to bp 210. The Alu sequence located downstream of exon 2 contains two internal sequences that are highly conserved in Alu repetitive sequences. The first is a sequence (GAGGCNGAGC) corresponding to the T-antigen binding sequence of the SV40 replication origin. The second is a symmetrical sequence (CCAGCCTGG) of no known function. This short symmetrical sequence is also present in both of the Alu sequences on either side of exon 14. Exons 14 and 15 are separated by a short intron that is almost entirely composed of Alu sequence, suggesting that exons 14 and 15 were possibly at one time a single exon and were divided by the insertion of an Alu element during evolution. This Alu sequence may have been directed to this position by the other Alu sequence located on the 5' end of exon 14 because retroposons have a tendency to integrate adjacaent to one another.

The 3' end of the retinoblastoma gene contains the usual polyadenylation signal sequence, AATAAA. One sequence (TGTGTTCT) located 32 bases downstream of this hexamer is equivalent to the conserved downstream consensus sequence

(YGTGTYY) described by McLauchlan et al. (1985). This sequence and surrounding bases compose the "G/T cluster" generally found in a region 30 nucleotides downstream of the polyadenylation signal sequence (Birnstiel et al., 1985).

The p4.7R probe also was used to screen genomic DNA isolated from the tumors of 50 unrelated individuals (40 retinoblastomas, 8 osteosarcomas, and 2 undifferentiated tumors of unknown cellular origin arising in patients with hereditary retinoblastoma), as described in more detail below. These DNA samples were digested with HindIII and analyzed by Southern blot hybridization using radiolabeled p4.7R as the probe. This analysis revealed three types of deviant patterns of the genomic DNA restriction fragments: totally absent fragments, representing apparent homozygous deletions; under-represented fragments, representing apparent heterozygous deletions; and fragments of altered size, reflecting either partial deletion or an alteration of a restriction site. At least 30% of the tumor DNA's exhibited one of these abnormalities. In comparison, Southern blot analysis of leucocyte DNA from 18 normal individuals showed a uniform pattern of restriction fragments.

Use

The cDNA and genomic sequences, e.g., those in p4.7R, can be used, according to the invention, to screen individuals for the presence of a mutated allele of the Rb gene. This

screening procedure will allow individuals having a risk of developing retinoblastoma--because of family history or a previous incidence of retinoblastoma in one eye--to determine the need for routine testing by the current ocular examination procedure. Only if the screening procedure determines that the individual possesses a mutant Rb allele will the examination procedure need to be conducted on a regular basis. Those with two normal Rb alleles can discontinue examination, as the risk of developing retinoblastoma in an individual with two normal copies of the Rb gene is approximately 1 in 20,000, or 0.005%, compared to a risk of 80%-90% if an individual has an Rb allele containing a mutation sufficient to inactivate the allele. Thus, a substantial percentage of individuals who are currently examined regularly are not actually at a greater risk than the general population: neither a family history of nor a previous incidence of retinoblastoma is conclusive evidence that an individual has the genetic predisposition to the disease. Therefore, such individuals, actually carrying two normal copies of the Rb gene, have been repeatedly undergoing the expensive and traumatic ocular examination procedure needlessly.

The screening procedure according to the invention includes: (1) testing a nucleic acid sample of a patient for large deletions in the Rb gene locus; (2) testing a nucleic acid sample of a patient for small deletions or point mutations in the Rb gene locus; and (3) testing a nucleic acid sample of

a patient for RFLPs linked to the Rb gene locus.

Detection of Large Deletions in the Rb Gene

The availability of DNA probes from the Rb gene provides a means of directly detecting genetic lesions that create retinoblastoma-predisposing alleles. Suitable probes include the entire normal retinoblastoma gene sequence, or fragments thereof consisting of 15 or more bases encoding a specific portion of the retinoblastoma gene. When performed by Southern blot and dot blot procedures, this analysis is generally limited to the study of those lesions that create gross structural changes in the Rb gene, such as deletion of many hundreds of base pairs.

The DNA for a Southern Blot or dot blot analysis is isolated from peripheral leucocytes or, if the patient has had a tumor in one eye, from the tumor. To examine leucocyte DNA, a 10 ml blood sample is obtained from the individual, and the genomic DNA is isolated from the leucocytes in the sample, according to standard techniques. This DNA is digested with a restriction endonuclease (e.g., HindIII), and the resulting fragments are separated on an agarose electrophoresis gel according to a physical property such as molecular shape or molecular weight. For the purposes of this invention, molecular shape is defined as the structural configuration of the molecule (e.g., linear, circular, double-stranded or single-stranded). The DNA in the gel is transferred to a

nitrocellulose filter by blotting. The filter is then probed with, e.g., radiolabeled p2AR3.8 and, separately, p2AR0.9, containing subfragments from p4.7R obtained by EcoR1 digestion. (The diagrams of the vectors p2AR3.8 and p2AR0.9 are shown in Figure 3.) In order to more precisely define the location of any abnormalities detected, two or more subfragment probes are used separately rather than the entire p4.7R insert probe. The autoradiograms of the probed filter generate the data necessary to construct a restriction map of the Rb locus in the somatic or tumor DNA of the tested individual.

This restriction map is compared with a control restriction map, determined by using the same restriction enzymes for digestion and the same probe. A suitable control is DNA obtained from an adenovirus-transformed retinal cell line or leucocyte DNA from a set of normal individuals. If the tested individual has an Rb allele containing a significantly large deletion, a restriction map of his DNA, compared with the control, will contain an additional band or bands, and/or a band or bands that have lost 50% of their intensity, caused by a change in the size, or total elimination, of one or more restriction fragments by the deletion in one allele at the Rb locus.

This screening procedure by Southern analysis will detect the existence of Rb alleles which have large deletions and are thereby non-functional. If this analysis indicates

that the tested DNA from an individual has a restriction map which is different from the control map, there is a high probability that the individual contains a non-functional, mutant Rb allele. The individual must be monitored closely thereafter for the development of retinoblastoma.

If the test restriction map appears identical to the control, a different screening procedure can be performed to determine if the individual possesses an Rb allele having a small deletion or point mutation. Small deletions and point mutations may be sufficient to inactivate the allele, but not prevent hybridization with a probe. An example of this screening procedure is outlined below.

Detection of Other Mutations in the Rb Gene

To examine a DNA sample of an individual for small deletions or point mutations in the Rb locus, both homologs of the Rb gene from said individual are cloned. The cloned alleles then can be tested for the presence of nucleic acid sequence differences from the normal allele, e.g., as represented by p4.7R, by one of the following two methods: (1) the nucleotide sequence of both the cloned alleles and p4.7R are determined and then compared, or (2) the RNA transcripts from p4.7R are hybridized to single stranded whole genomic DNA from an individual to be tested, and the resulting heteroduplex is treated with Ribonuclease A (RNase A) and run on a denaturing gel to detect the location of any mismatches. In

more detail, these methods can be carried out according to the following procedure.

The alleles of the Rb gene in an individual to be tested are cloned using conventional techniques. A common method, for example, employs the bacteriophage vector EMBL3 (Frischauf et al., 1983, J. Mol. Biol., Vol. 170, pp. 827-). A 10 ml blood sample is obtained from the individual. The genomic DNA isolated from the cells in this sample is partially digested with MboI to an average fragment size of approximately 20 kb. Fragments in the range from 18-21 kb are isolated. The resulting MboI-ended fragments are ligated into the EMBL3 vector DNA which has been completely digested with BamHI, treated with alkaline phosphatase, and heated to 68°C for 10 minutes to disrupt the cohesive ends. This ligation mix is used in an in vitro lambda packaging reaction, and the packaged phage are amplified by growing a plate stock. (This cloning technique is described generally in Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Publications, pp. 256-293.)

Approximately 5 x 10^5 plaque forming units (pfu) from this plate stock are then screened with radiolabeled p4.7R by hybridization and autoradiography. Plaques which show hybridization to the p4.7R probe are plaque-purified and rescreened according to the above procedure. Positive plaques from the rescreening are isolated and used to prepare DNA

putatively containing Rb alleles from the individual.

The MboI genomic inserts in these isolated EMBL3 vector DNA samples are tested for the location of the sequences homologous to p4.7R by Southern analysis. DNA samples containing the entire Rb gene region are selected, and the appropriate restriction fragments containing the Rb gene from these samples are subcloned into a suitable vector, such as pUC9. These subclones thus contain copies of one or both Rb alleles from the DNA of the individual to be tested. To determine if both alleles are represented, the initial phage isolates are tested for the existence of restriction polymorphisms. These subcloned alleles are then examined for differences from p4.7R by one of the following techniques.

First, the nucleotide sequence of the normal Rb gene in p4.7R is determined. Restriction fragments of approximately 500 base pairs (bp) from p4.7R are subcloned into an M13mp8 phage vector and sequenced by the dideoxy technique (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, Vol. 74, pp. 5463-). A composite sequence of the Rb gene then can be assembled from these individual subclone sequences. The complete sequence of the normal retinoblastoma gene and flanking sequences is shown in Fig. 5 and Fig. 6.

The isolated Rb gene alleles are sequenced according to the following procedure. Restriction fragments (~ 2kb) of the allele are subcloned into the M13mp8 vector, and short

stretches (~500 bp) are sequenced individually using small restriction fragments isolated from p4.7R as the primers in the dideoxy sequencing reactions. The composite nucleotide sequence of the isolated allele then can be constructed from these individually-primed sequences. This sequence is compared directly with the sequence of the normal Rb gene, determined from p4.7R, to reveal any deletions or point mutations in the isolated allele.

An alternative method of comparing the allelic DNA with the normal Rb gene employs RNase A to assist in the detection of differences between the p4.7R sequence and the allele sequence. This comparison is performed in steps using small (~500 bp) restriction fragments of p4.7R as the probe. First, p4.7R is digested with a restriction enzyme(s) that cuts the Rb gene sequence into fragments of approximately 500bp. These fragements are separated on an electrophoresis gel, purified from the gel and cloned individually, in both orientations, into an SP6 vector (e.g., pSP64 or pSP65; Melton et al., 1984, Nucleic Acids Res., Vol. 12, pp. 7035-). The SP6-based plasmids containing inserts of p4.7R fragments are transcribed in vitro using the SP6 transcription system, well known in the art, in the presence of [α-^{32}P]GTP, generating radiolabeled RNA transcripts of both strands of the cDNA of the Rb gene.

Individually, these RNA transcripts are used to form

heteroduplexes with the allelic DNA, as described by Myers et al., 1985, Science, Vol. 230, pp. 1242-46, the teachings of which are incorporated herein by reference. Mismatches that occur in the RNA:DNA heteroduplex, owing to sequence differences between the p4.7R fragment and the Rb allele subclone from the individual, result in cleavage in the RNA strand when treated with RNase A. Such mismatches can be the result of point mutations or small deletions in the individual's Rb allele. Cleavage of the RNA strand yields two or more small RNA fragments, which run faster on the denaturing gel than the RNA probe itself.

In the RNAse A technique, radiolabeled Rb gene RNA is hybridized to single strands of an individual's Rb alleles which have been cloned into a vector. The RNase A technique is advantageous, however, because it also can be used without having to clone the Rb alleles. Preferably, genomic DNA is isolated from blood cells of the individual to be tested, and this genomic DNA is hybridized directly with the radiolabeled Rb RNA probes to determine sequence differences from the normal Rb gene. Specifically, 5 µg of isolated, total genomic DNA is resuspended with the labeled RNA probe in 30 µl of hybridization buffer (80% formamide, 40mM Pipes pH6.4, 0.4M NaCl, and 1mM EDTA), and this hybridization mix is treated at 90°C for 10 minutes to denature the DNA. The mixture then is cooled slowly to 45°C and incubated at this temperature for 10

hours to allow hybridization of the RNA probe to the single-stranded DNA copies of the Rb allele. After hybridization, RNase A treatment and electrophoresis are performed as described by Myers et al., supra. Mismatches between the RNA probe and the genomic copies of the individual's Rb alleles are then readily detected.

Detection of RFLPs Linked to the Rb Gene

The inheritance of a retinoblastoma- predisposing defect can be traced by following its co-inheritance with DNA polymorphisms in a pedigree analysis.

The gene map shown in Figure 2 was used to develop nucleic acid probes useful for retinoblastoma diagnosis. To do so, the bacteriophage DNA corresponding to the human inserts were subcloned in the plasmid vector "Bluescribe" (Stratagene). Fifteen single-copy DNA fragments from the gene, ranging in size from 500 bp to 2000 bp, were subcloned. These sequences are scattered over the 200 kb of the mapped region. Subcloned DNA fragments were separated from vector sequences by digestion of plasmid DNA with one or more restriction endonucleases, electrophoresis through a 0.6% low-melting-point agarose gel, and purification by chromatography using an Elutip-d column (Schleicher and Schuell). Purified DNA fragments were radiolabeled with ^{32}P-dCTP (New England Nuclear) by the random primer technique using the Klenow fragment of DNA polymerase I.

Restriction fragment length polymorphisms (RFLP's) were discovered by digesting genomic DNA isolated from six normal individuals with 33 different restriction enzymes. The DNA fragments resulting from the 198 separate digests were separated on a 0.8% agarose electrophoresis gel according to molecular shape or molecular weight. The DNA was transferred to nitrocellulose filters and hybridized with single copy DNA probes purified from the retinoblastoma gene according to published methods (T.P. Dryja et al., 1986, Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 7391-94).

Of the 15 single-copy probe fragments, only five reveal RFLP's. Four of the polymorphisms appear to be the result of minor alterations (perhaps single base changes) in the recognition sequence of a restriction endonuclease (KpnI, XbaI, MboII, or Tth111I). The fifth polymorphism reflects variability in the number of tandem repeats of a 50 base pair sequence. The location of the DNA polymorphisms are shown in the map in Figure 2 (vertical arrows above the map). The location of the polymorphic MboII site(s) has not been determined precisely but is located at approximately 175 kb on this map. The frequencies of alleles which correspond to particular DNA polymorphisms are indicated in Table 1.

In order to demonstrate the utility of these probes to detect the presence of retinoblastoma-predisposing alleles in humans, twenty pedigrees with hereditary retinoblastoma were

analyzed. DNA was extracted from leucocyte nuclei of venous blood from available family members according to the method of Kunkel et al., 1977, Proc. Natl. Acad. Sci. USA, Vol. 74, pp. 1245-49, hereby incorporated by reference. For analysis of a kindred with a given RFLP, DNA from the available family members was digested with the appropriate restriction endonuclease. The resulting fragments were separated by agarose-gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled probe.

In these families, the inheritance of alleles determined by the DNA polymorphisms within the retinoblastoma gene were traced and compared to the inheritance of the retinoblastoma-predisposing trait. For example, consider the polymorphism detected by probe p68RS2.0 (see Table 1). When genomic DNA is digested with the restriction enzyme RsaI, this probe hybridizes to allelic DNA fragments of different lengths. The size of these fragments ranges from 1.5 kb to 2.0 kb with intervals of approximately 50 bp. The DNA sequence of the 2.0 kb genomic fragment cloned in p68RS2.0 has a 50 to 53 bp segment which is repeated approximately 30 times (Table 2). This 53bp segment can be used as a probe in these analyses. A portion of the repeated sequence has homology to core sequences of VNTR's (Variable Number of Tandem Repeat) reported elsewhere (Y. Nakamura et al., 1987, Science, Vol. 235, pp. 1616-22). (The 11 bp sequence shown in Table 2 above the repeat unit

TABLE 1
DNA POLYMORPHISMS
IDENTIFIED WITHIN THE RETINOBLASTOMA GENE

DNA PROBE	RESTRICTION ENDONUCLEASE	MAP LOCATION[1]	ALLELES SIZE (Kb)[3]	FREQUENCY[4]
p68RS2.0	RsaI	142-143 kb	2.00	0.13
			1.95	0.02
			1.90	0.07
			1.85	0.07
			1.80	0.35
			1.75	0.20
			1.65	0.09
			1.50	0.09
p88PR0.6[5]	XbaI	120 kb	7.0	0.55
			5.5	0.45
p35R0.6	Tth111I	195 kb	4.95	0.20
			4.35	0.80
p2P0.3	MboII	175 kb[2]	1.0	>.90
			0.8	<.05
			0.6	<.05
			0.3	<.05
p95HS0.5	KpnI	25 kb	12.0	0.95
			8.0	0.05

[1]The map location of each polymorphic site refers to the position on the restriction map of the gene shown in Figure 1.

[2]The location of the MboII site is approximate, since the precise position of this site within the map is not yet known.

[3]The allele sizes were calculated from several indepedent measurements using HindIII fragments of lambda phage DNA as a standard.

[4]Allele frequencies are based on a population of 40-60 unrelated individuals.

[5]p88PRO.6, p35RO.6, p2PO.3 and p95HS0.5 are probes isolated from other regions of the retinoblastoma gene, shown in Fig. 2.

represents the core sequence reported for some VNTR's observed by Nakamura et al.) Because such tandemly repeated sequences tend to be genetically unstable, the number of repeats is highly variable. Eight distinct alleles at this site have been detected, and more may exist. Because of this number of common alleles, seventy-five percent of unrelated individuals are heterozygous for this polymorphism. The high frequency of heterozygosity makes this polymorphism extremely useful. (In Table 2, the brackets "(" and ")" denote regions of variability within the repeat unit; and the bases underlined above and below the bracketed regions denote possible alternate bases for those regions of variability.)

TABLE 2

SEQUENCE OF THE REPEAT UNIT WITHIN p68RS2.0

```
                                    GGGNNGTGGGG
          TG       A                |||||||  ||          AG
CGTGTTGAA( A)ACAC(C)TCCCCAATGCTGGAGGTGAGGTTTGGT(AGAAG)ATGACT
           G
```

Fourteen retinoblastoma families carried constellations of alleles at this DNA polymorphism. This variation allows an examination of the frequency of co-inheritance of this site with the retinoblastoma-predisposing trait. For example, genomic DNA of individuals in these families was digested with Rsa I and the co-inheritance of any one Rsa I fragment with defective Rb alleles determined.

Sequence Analysis

RFLP analysis can reveal only those sequence variations that give rise to a restriction fragment that is detectably different on a Southern blot from restriction fragments characterizing the normal nucleic acid. Most such detectable polymorphisms result from DNA sequence variation within a restriction endonuclease recognition site. These sites are rare, and finding them requires a laborious and expensive screening process in which genomic DNAs from several unrelated individuals are digested with as many as 50 different restriction enzymes. The fraction of all genomic DNA sequence polymorphisms (DSPs) at a specific locus that can be detected as RFLPs is small and depends on the number of enzymes used for screening; generally 90% or more of the DNA sequence polymorphism in the human genome is not within reach of RFLP-based analysis. Tens or even hundreds of potentially useful DSPs may exist within or near most disease-causing genes, but often only a few and sometimes none of these DSPs are detectabale as RFLPs. Fig. 4 shows a scale map of the 200 kilobase genomic region that includes the 27 exons of the human retinoblastoma gene. The 27 exons make up a 4.7 kilobase transcript.

Fig. 7 shows the locations of the DNA sequence polymorphisms (DSPs) identified in this gene. Polymorphisms identified by the name of a restriction enzyme are RFLPs;

polymorphisms RB1.2, RB1.3, RB1.20 and RB1.26 are not detectable as RFLPs, and were found by PCR-amplification and direct sequencing, as described below. The 200 kilobase genomic region was isolated in a series of overlapping inserts from 35 distinct recombinant bacteriophage lambda clones. Exon-containing segments were subcloned into bluescribe plasmid cloning vectors (Stratagene, Inc.). Initial sequencing of cloned plasmid inserts was carried out using conventional methods for plasmid sequencing. Based on this genomic sequence, pairs of 20-base oligonucleotide primers were synthesized so that numerous regions 320 - 1200 bp in size could be amplified from genomic DNA by the polymerase chain reaction (PCR) method of Mullis et al.

For each amplification reaction, from 200 ng to 1.0 ug of genomic DNA was prepared in a reaction buffer containing 20 mM Tris (pH 8.4 or pH 8.6), 30 ug/ml bovine serum albumin, 300 mM to 7.5 mM, 10-50 pM of each oligonucleotide primer, and 1 unit of Taq polymerase (Perkin-Elmer Cetus). Optimal $MgCl_2$ concentrations and pH of the PCR reactions varied depending on the primer pair. PCR-amplification (30-35 rounds) was carried out following a cycle of 10 seconds at 94°C (denaturation), 10 seconds at 42-55°C (annealing), and 30 seconds at 70°C (Polymerization) using a programmable thermal cycler (Ericomp Corp., San Diego). All times are based on sample temperature rather than heat-block temperature, and do not include 'ramping

time' for the heat block. Optimal annealing temperatures varied for each primer pair. This protocol allowed PCR-amplification of regions as large as 2500 bp. Genomic DNA from 9-20 individuals was amplified for each region and screened for DSP.

In order to detect and utilize a greater fraction of the existing DNA sequence polymorphism in the retinoblastoma gene, we applied techniques of polymerase chain reaction (PCR), generally as described by K.B. Mullis et al., 1987, Methods Enzymol., Vol. 155, pp. 335-51, and direct sequencing, generally as described by C. Wong et al., 1987, Nature, Vol. 330, 384-86, to analyse normal allelic variation at this locus. Oligonucleotide primers were synthesized to amplify regions from the gene that varied in size from 320 - 1200 bp. Amplification and sequencing were carried out on DNA from at least 9 unrelated individuals for all regions screened, though for many regions 15 or more individuals were analyzed. In most cases, primer pairs were derived from intronic sequences that flanked one of the 27 exons of the gene, such that the PCR-amplified region contained both intron and exon sequences.

The results of this screening process are shown in Tables 3 and 4. Amplified DNA sequences were compared to one another and checked against sequence data from previously cloned plasmid inserts derived from the same region. Bases obscured by technical artifacts or other ambiguities were not

tabulated. Of 3712 bp of genomic DNA sequence screened at this locus, four sequence variations were identified (Table 3; map locations are shown in Fig. 7). All four variations were found in introns; of these, one is likely a rare variant (found in only 1 or 15 individuals sequenced), and three represent bona fide DNA sequence polymorphisms. A representative example (RB1.3) is illustrated in Fig. 7. This polymorphism occurs near exon 3 of the retinoblastoma gene. Neither form of the polymorphic sequence forms the recognition site of a known restriction enzyme, and hence this DSP is not detectable as an RFLP. Among a total of 82 genetically distinct (from unrelated individuals) alleles examined, no other base was observed at this site.

Fig. 8 illustrates the inheritance of the polymorphism RB1.3 in a retinoblastoma-prone family. Oligonucleotide primers (see Table 4) were used to PCR-amplify a 530 bp region of the human retinoblastoma gene that includes exon 3. The amplified fragment was sequenced by the methods described below. The sequence surrounding the polymorphism is written at the left side of the figure, read 5' to 3' from bottom to top, and the polymorphic bases are identified by adjacent tic marks.

The details of the analysis were as follows. Prior to sequencing, all PCR-amplified DNA samples were treated with proteinase-K and extracted with phenol/chloroform. High molecular weight DNA was separated from unused dNTPs and

oligonucleotide primers by column purification through sepharose CL-6B (Pharmacia). 250-400 ng of double-stranded PCRF-amplified template was combined with 1-2 pM of (^{32}P) end-labeled sequencing primer, and heat-denatured for 3 minutes at 96°C. This primer-template mixture was added to a buffer containing: $MgCL_2$ (2.5 mM), Tris-HCL pH 7.5 (5 mM), 6 units Sequenase (U.S. Biochemical) and dithiothreitol (3 mM), and divided into 4 reaction mixtures each containing all four deoxynucleotides (32 uM each) and one dideoxynucleotide (5 uM). This mixture was immediately incubated for 5 minutes at 37-42°C, and polymerization was stopped with a 0.37% EDTA stop buffer. Prior to loading on sequencing gels, the samples were heat denatured at 96°C for 2 minutes. Conventional 0.4 mm thick, 6% polyacrylamide sequencing gels were used, and autoradiography was typically for 12-24 hours without an intensifying screen.

Fig. 9 illustrates segregation of the DSP RB1.3 in three families with hereditary retinoblastoma. Alleles are shown beneath the symbol for each person. Affected individuals are indicated by filled symbols. In family RB-32, the (-) allele is the result of an intragenic deletion. By subsequent Southern blotting studies, the deletion was found to extend from exon 2 to exon 17. Based on these results, it can be predicted that the unaffected members of family RB-32 who carry the (G,-) genotype are also carriers of the mutation.

The DSPs we have detected are valuable genetic markers for our studies of hereditary retinoblastoma. In its hereditary form, a predisposition to the disease is passed from affected individuals to their offspring as a dominant trait with 90% penetrance. It can be seen from Fig. 8 that the affected father, who has passed the disease to two children, is heterozygous for RB1.3. Both affected children received the G allele, while the unaffected child inherited the allele marked by an A at this polymorphic site. In this family, then, inheritance of the G allele from the affected parent is in phase with and diagnostic for the disease-predisposing phenotype. Fig. 9 shows our analysis of three other retinoblastoma-prone families using RB1.3. Inheritance of the polymorphic markers we describe here has followed the expected Mendelian pattern in every family examined so far. No cross-overs were observed between the polymorphic sites and the retinoblastoma-predisposing trait in any of the pedigrees. This follows our expectations since the polymorphisms are within the disease gene. In family RB-32, an intragenic deletion in one copy of the RB gene, presumably causing the predisposition to the tumor, was identified by Southern blotting (data not shown). The deletion includes the region surrounding RB1.3, and hence carriers of the disease-predisposing allele are genotypically hemizygous for the A allele (A,-). Two unaffected members of pedigree RB-32

are carriers for the disease-predisposition, based on analysis of RB1.3 (see Fig. 9). More happily, Fig. 9 shows that the other unaffected children in pedigree RB-32, as well as those in pedigrees RB-36 and RB-50, are not carriers of the cancer-predisposition and therefore will not pass the disease on to their children. These results highlight the diagnostic value of this class of human genetic markers that were heretofore unavailable for this purpose.

The data we present may also be used to estimate the level of heterozyygosity in the human genome from a novel perspective. Previous estimates based on restriction enzyme screening may be subject to a bias because the sequences recognized by these enzymes do not necessarily reflect a random sampling. It is likely that a substantially higher level of polymorphism occurs at CpG pairs than elsewhere. This is reflected by the relatively high proportion of RFLPs revealed by such enzymes as Msp I (CCGG) and Taq I (TCGA). The method we describe is not subject to this bias. From the results of our screening, it can be calculated that genomic heterozygosity at this locus is approximately h=0.00039. If only the intron seqeunces are considered, this estimate increases to h=0.00070. These estimates are below the predictions of others, and may reflect the absence from our methods of the bias described above. However, an analogous calculation of heterozygosity ($0.00044 < h < 0.00087$) based instead on our

initial RFLP screening is also below the estimates of others and is quite consistent with our estimate based on direct sequencing. It seems likely that the human retinoblastoma gene is intrinsically less polymorphic than many other regions of the genome. Although mutations in this gene are known to be early events in the formation of several types of cancer, it is unclear why polymorphism at this locus may have been selected against in human evolution.

The approach for detecting DSPs demonstrated here has several advantages over conventional RFlP-based screening. As we have argued, DSP screening by amplification and direct sequencing could increase by an order of magnitude the number of available polymorphic markers at any cloned locus. This technique encompasses and supercedes restriction enzyme-based screening since RFLPs and VNTRs may also be detected. As the only requirement for utilization of such markers is knowledge of a unique set of amplification primer sequences and of the polymorphism itself, publication of a polymorphism immediately makes it available to all readers. Hence, problems and delays associated with the physical transfer of plasmid DNAs between laboratories are avoided, and the costs of maintaining plasmid repositories will be ultimately reduced. In addition, rapid analysis of these polymorphic markers can be carried out on a large scale with the use of allele-specific oligonucleotide probes for direct hybridization to amplified DNA. Finally,

based on our experience with both strategies at the same locus, we found the expense and effort required to locate DSPs by either method to be comparable.

Treatment of Patients Having a Defective Rb Gene

In addition to screening, the invention includes polypeptide therapy for those individuals determined to contain a defective Rb allele, and who therefore are at risk of developing retinoblastoma.

To prevent the formation of retinoblastoma in these individuals, the Rb polypeptide is administered therapeutically in an amount sufficient to inhibit retinoblastoma tumor formation or growth (anti-retinoblastoma-forming amount). An anti-retinoblastoma-forming dosage of the Rb polypeptide is 1 to 500 µg/kilogram of body weight/day. The Rb protein can be administered by injection with a pharmacologically acceptable carrier, either alone or in combination with another agent. Acceptable pharmacological carriers are those which dissolve the Rb polypeptide or hold it in suspension, and which are not toxic to the extent of permanently harming the patient. Preferred are aqueous solutions of salts or non-ionic compounds such as sodium chloride or glucose, most preferably at an isotonic concentration. Other agents may be present provided that they do not interfere with the action of the Rb polypeptide. Those skilled in the art will know, or will be able to ascertain with no more than routine experimentation,

particular pharmacological carriers for this composition.

Rb polypeptide suitable for therapy can be prepared by any one of the following three conventional procedures. First, the Rb polypeptide can be produced by cloning the Rb cDNA from p4.7R into an appropriate mammalian expression vector, expressing the Rb gene product from this vector in an in vitro expression system, and isolating the Rb polypeptide from the medium or cells of the expression system. General expression vectors and systems are well known in the art.

Second, the Rb polypeptide can be produced using protein chemistry techniques, wherein the specific amino acid residues are joined together synthetically in the appropriate sequence.

Third, naturally occurring Rb protein can be isolated from total protein samples by affinity chromatography. Antibodies specific for the Rb protein are prepared by standard procedures (see below) and coupled to an inert matrix, which is then used to selectively bind the Rb proteins.

Immunodiagnosis of Retinoblastoma

This invention also includes methods for determining whether a particular tumor is the result of an Rb gene abnormality. Since osteosarcomas and certain undifferentiated tumors can result from detectable lesions in the Rb gene, immunodiagnosis can be used to aid in the diagnosis of such tumors.

In order to produce anti-Rb antibody, a rabbit is immunized with either naturally occurring Rb protein or Rb polypeptide produced as described above. The anti-Rb antibody generated is then labeled, e.g., radioactively, fluorescently, or with an enzyme such as alkaline phosphatase. The labeled antibody is used to determine whether human tumors are of defective Rb gene origin. This can be carried out using any conventional technique. For example, the tumor sample can be liquified and tested against the labeled antibody using a conventional ELISA (Enzyme-linked immunosorbent assay) format. Alternatively, human tissue samples (e.g., biopsy samples) can be tested for expression of the retinoblastoma protein by other immunological techniques, see e.g., I. Roitt, Interaction of Antigen and Antibody, In Essential Immunology, Fifth edition, Boston: Blackwell Scientific Publications, 1984, pp. 145-75.

Immune complexes will be detected in tumor samples which have antigens (e.g., retinoblastoma polypeptide) reactive with anti-Rb antibody. Tumors which lack these antigens presumptively have a defect (e.g., mutation or a deletion) in the retinoblastoma gene.

Deposits

Plasmids p2AR3.8 and p2AR0.9 were deposited on July 17, 1987 with the American Type Culture Collection, Rockville, Maryland, and assigned ATCC accession numbers 40,241 and 40,242, respectively.

The Applicants represent the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendancy of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicant acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

Other embodiments are within the following claims.

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Table 4. Polymorphic sequences characterized and primer pairs used for PCR-amplification

	Polymorphic Sequence	Allele Frequency	Amplification Primer Pair	Fragment Size	Location
RB1.2:	TAAAATAAGATCTTAAAG	>95%	5'-AAGTGTAATGTTTTTCTAAG-3'	431 bp	124 bp from 5' end of exon 2
	TAAAATAAGA CTTAAAG	< 5%	5'-TAGCAGAGGTAAATTTCCTC-3'		
RB1.3:	CAGAATTCGTTTCCTTTT	73%	5'-TTCAAATATATGCCATCAGA-3'	530 bp	43 bp from 3' end of exon 3
	CAGAATTCATTTCCTTTT	27%	5'-GCTTACACATGAATAGTGAGAG-3'		
RB1.20:	GATTT(CTTT)$_n$CCTTTT n=14-26	N/D	5'-AATTAACAAGGTGTGGTGG-3'	550-600 bp	54 bp from 3' end of exon 20
			5'-CTTGTAATATGCCTCATAAT-3'		
RB1.26:	ATTTTTTTAATCTGCAGT	85%	5'-ATTCAGTGAAGATATCTAAT-3'	683 bp	10 bp from 5' end of exon 26
	ATTTTTTAAATCTGCAGT	15%	5'-TAGTTCCTCTTTGTAGTTCT-3'		

Shown are the sequences and locations of the polymorphic sites and their immediate flanking regions. Also shown are the oligonucleotide primers used to amplify these sequences from human genomic DNA. 'Fragment size' refers to the PCR-amplified product. Allele frequencies are based on analysis of the following numbers of individuals (of mixed North American descent): (RB1.2)-15; (RB1.3)-41; (RB1.20)-14; (RB1.26)-27. Accurate allele frequencies for RB1.20 have not yet been determined, as numerous alleles were found.

Table 3

DNA sequence polymorphisms detected by direct sequencing

	Base Pairs Screened	Polymorphisms
Introns	2072	4
Exons	1640	0
Totals	3712	4

DNA sequence polymorphisms found by direct sequencing of 13 separate PCR-amplified regions from the human retionoblastoma locus. DNA samples from a minimum of 9 unrelated individuals were examined for all bases screened. Bases that could not be scored unambiguously were excluded from this tabulation.

Claims

1. Purified nucleic acid comprising a human retinoblastoma gene, or a fragment thereof comprising 15 or more bases, said nucleic acid being less than 100kb in size.

2. A vector comprising the nucleic acid of claim 1.

3. A cell transformed with DNA encoding retinoblastoma polypeptide or a fragment thereof.

4. The nucleic acid of claim 1, wherein said nucleic acid hybridizes specifically to said retinoblastoma gene under hybridizing conditions.

5. An isolated polypeptide encoded by the nucleic acid of claim 1.

6. An antibody produced to the polypeptide of claim 5.

7. An antibody produced to naturally occurring retinoblastoma polypeptide.

8. A method of detecting large deletions in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising the steps of:

hybridizing a nucleic acid sample from said patient with a probe specific for the retinoblastoma gene, and

determining the ability of said probe to hybridize to said nucleic acid,

wherein lack of hybridization to said nucleic acid indicates the presence of a large deletion in said gene.

9. A method of detecting large deletions in the retinoblastoma gene of a human patient that may predisposing said patient to retinoblastoma, comprising the steps of:

generating nucleic acid fragments from a sample of said patient,

separating said fragments according to a determined physical property of said fragments,

hybridizing a probe specific for the retinoblastoma gene to said fragments,

detecting hybrids of said probe and said fragments, and

comparing said hybrids to hybrids detected from the hybridization of said probe and separated nucleic acid fragments from a normal retinoblastoma gene,

wherein the absence of hybrids, or the smaller size of said hybrids from the sample of said patient is an indication of large deletions in the retinoblastoma gene of said patient.

10. The method of claim 8 or 9, wherein the probe specific for the retinoblastoma gene is the cloned DNA in p4.7R, or a fragment thereof.

11. The method of claim 9, wherein the physical property is molecular weight.

12. A method of detecting small deletions or point mutations in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising the steps of:

determining the nucleotide sequence of a retinoblastoma allele, or subregion thereof, from said patient, and

comparing said nucleotide sequence with the nucleotide sequence of a retinoblastoma allele or region thereof from a person not afflicted with retinoblastoma.

13. A method of detecting small deletions or point mutations in the retinoblastoma gene of a human patient predisposing said patient to retinoblastoma, comprising detecting mismatches between a nucleic acid sample from said patient and a detectable probe specific for the retinoblastoma gene from a person not afflicted with retinoblastoma, wherein mismatches are an indication of small deletions or mutations in the retinoblastoma gene of said patient.

14. A method of diagnosing predisposition of a human patient to retinoblastoma, comprising detecting the co-inheritance of retinoblastoma alleles of said patient with DNA polymorphisms in a pedigree analysis.

15. A method of detecting genetic polymorphisms, in the retinoblastoma gene of a human patient, that predispose said patient to retinoblastoma, comprising the steps of:

generating nucleic acid fragments from a sample of said patient,

separating said fragments according to a determined physical property of said fragments,

hybridizing a detectable nucleic acid probe capable of hybridizing to the wild type retinoblastoma gene to said fragments,

detecting hybrids of said probe and said fragments, and

comparing said hybrids to hybrids detected from the hybridization of said probe and separated nucleic acid fragments from a sample of a parent of said patient,

wherein the co-inheritance of specific genetic polymorphisms with the retinoblastoma gene is an indication of the predisposition of said patient to retinoblastoma.

16. The method of claim 15, wherein said physical property is molecular weight.

17. A method of treating a human patient having a defective retinoblastoma gene comprising administering to said patient an anti-retinoblastoma-
forming amount of the retinoblastoma polypeptide.

18. A composition suitable for treating a human patient having a defective retinoblastoma gene, comprising retinoblastoma polypeptide and a pharmacologically acceptable carrier therefor.

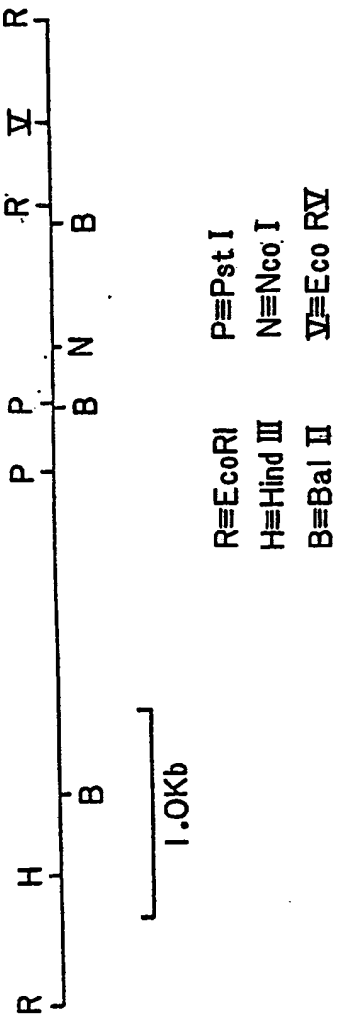
19. A method of detecting the presence, in a tumor sample, of a protein the absence of which is associated with a neoplasm, said method comprising producing an antibody to said protein, contacting said antibody with said tumor sample, and detecting immune complexes as an indication of the presence in said tumor sample of said protein.

20. A method of detecting the presence of the retinoblastoma protein in a tumor sample from a human patient, comprising the steps of:

contacting said tumor sample with an antibody which specifically reacts with the retinoblastoma protein, and

determining whether immune complexes are formed with said antibody, the formation of said immune complexes being an indication that the tumor is not retinoblastoma and the absence of immune complexes indicating that the tumor is retinoblastoma.

21. The method of claim 20, wherein said antibody is a monoclonal antibody.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B
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N
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B
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V
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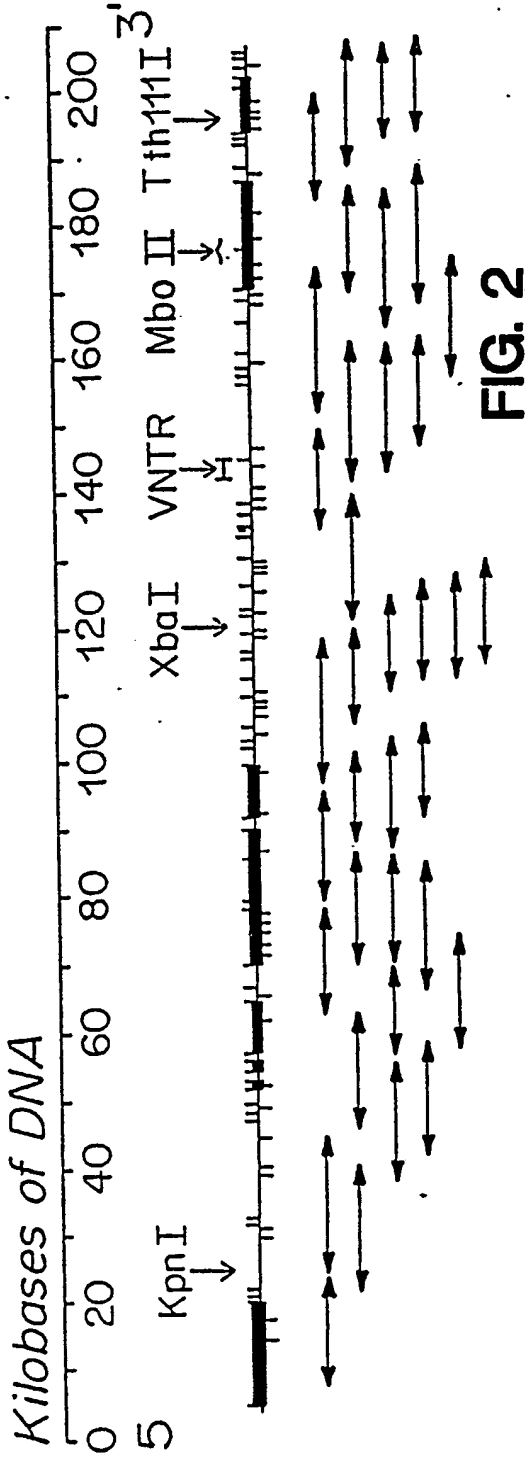
V≡Eco RV

FIG. 1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20
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60
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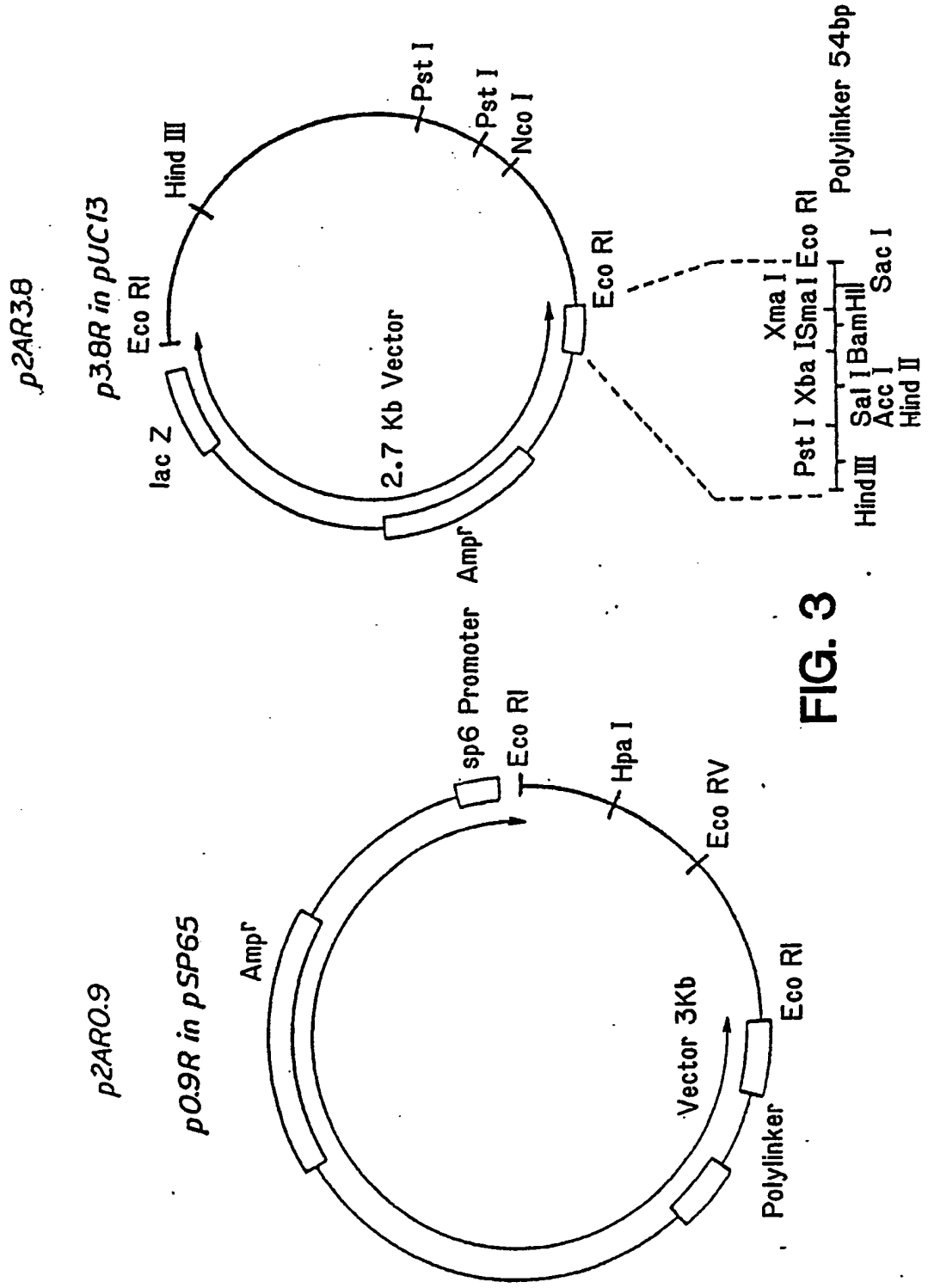
FIG. 2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Xma I
Sma I
Eco RI
Polylinker 54bp
Sac I
Pst I
Xba I
Sal I
BamHI
Acc I
Hind II
Hind III
p2AR0.9
p0.9R in pSP65
sp6 Promoter
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Eco RI
Polylinker

FIG. 3

FIG. 4

MAP OF THE RETINOBLASTOMA GENE

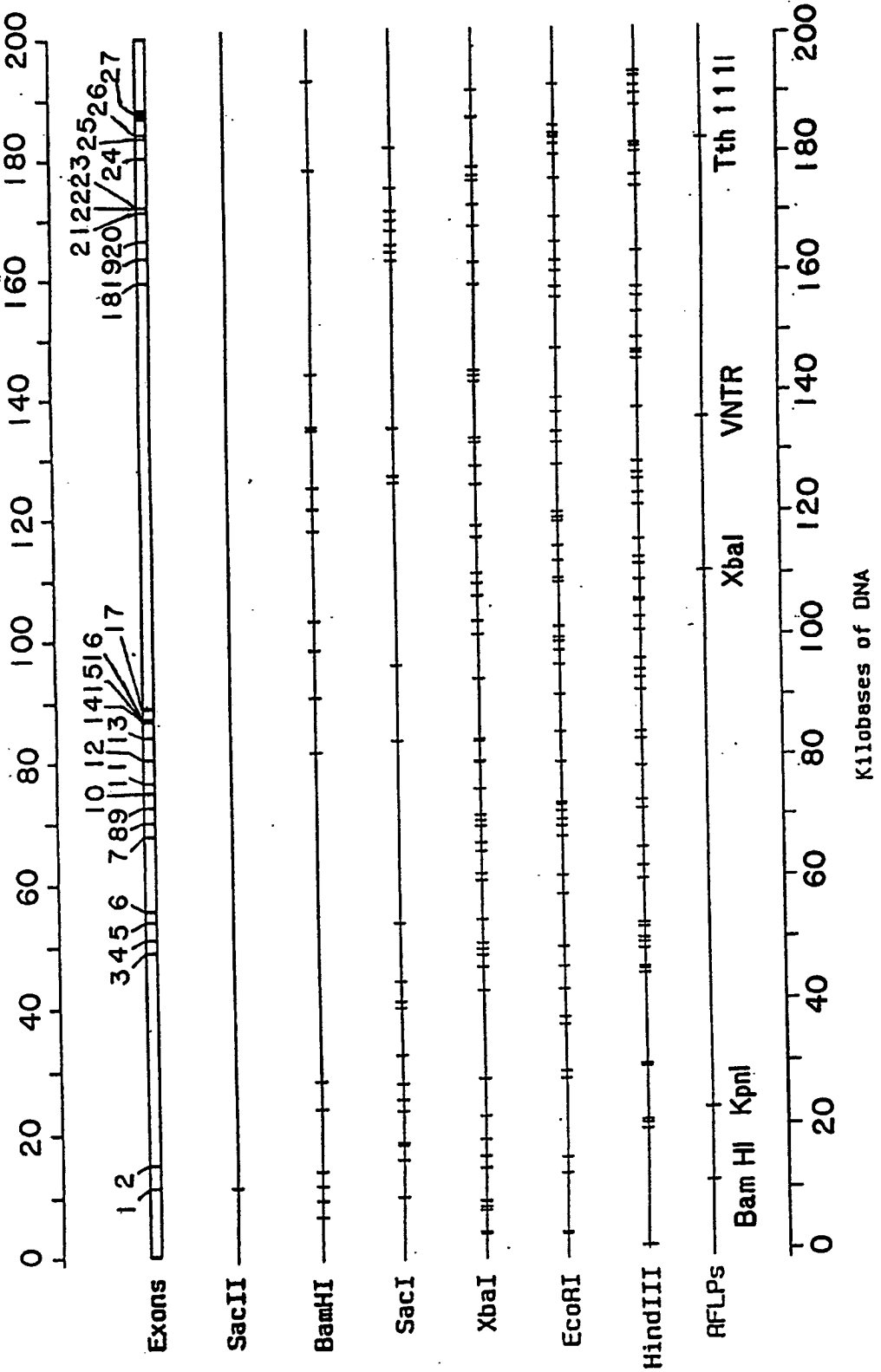

FIG. 5-1

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    S  V  H  K  F  F  N  L  L  K  E  I  D  T

421 AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG 462
    S  T  K  V  D  N  A  M  S  R  L  L  K  K

463 TAT GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA 504
    Y  D  V  L  F  A  L  F  S  K  L  E  R  T

505 TGT GAA CTT ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT 546
    C  E  L  I  Y  L  T  Q  P  S  S  S  I  S

547 ACT GAA ATA AAT TCT GCA TTG GTG CTA AAA GTT TCT TGG ATC 588
    T  E  I  N  S  A  L  V  L  K  V  S  W  I

589 ACA TTT TTA TTA GCT AAA GGG GAA GTA TTA CAA ATG GAA GAT 630
    T  F  L  L  A  K  G  E  V  L  Q  M  E  D

631 GAT CTG GTG ATT TCA TTT CAG TTA ATG CTA TGT GTC CTT GAC 672
    D  L  V  I  S  F  Q  L  M  L  C  V  L  D

673 TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC AAA GAA CCA 714
    Y  F  I  K  L  S  P  P  M  L  L  K  E  P

715 TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA ACA 756
    Y  K  T  A  V  I  P  I  N  G  S  P  R  T

757 CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA 798
    P  R  R  G  Q  N  R  S  A  R  I  A  K  Q

799 CTA GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA 840
    L  E  N  D  T  R  I  I  E  V  L  C  K  E

841 CAT GAA TGT AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA 882
    H  E  C  N  I  D  E  V  K  N  V  Y  F  K

883 AAT TTT ATA CCT TTT ATG AAT TCT CTT GGA CTT GTA ACA TCT 924
    N  F  I  P  F  M  N  S  L  G  L  V  T  S

925 AAT GGA CTT CCA GAG GTT GAA AAT CTT TCT AAA CGA TAC GAA 966
    N  G  L  P  E  V  E  N  L  S  K  R  Y  E

967 GAA ATT TAT CTT AAA AAT AAA GAT CTA GAT GCA AGA TTA TTT 1008
    E  I  Y  L  K  N  K  D  L  D  A  R  L  F

1009 TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT TCT ATA GAC AGT 1050
     L  D  H  D  K  T  L  Q  T  D  S  I  D  S

1051 TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT GAT GAA 1092
     F  E  T  Q  R  T  P  R  K  S  N  L  D  E

1093 GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT 1134
     E  V  N  V  I  P  P  H  T  P  V  R  T  V

1135 ATG AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA 1176
     M  N  T  I  Q  Q  L  M  M  I  L  N  S  A

1177 AGT GAT CAA CCT TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC 1218
     S  D  Q  P  S  E  N  L  I  S  Y  F  N  N
```

FIG. 5-2

```
1219  TGC ACA GTG AAT CCA AAA GAA AGT ATA CTG AAA AGA GTG AAG  1260
        C   T   V   N   P   K   E   S   I   L   K   R   V   K

1261  GAT ATA GGA TAC ATC TTT AAA GAG AAA TTT GCT AAA GCT GTG  1302
        D   I   G   Y   I   F   K   E   K   F   A   K   A   V

1303  GGA CAG GGT TGT GTC GAA ATT GGA TCA CAG CGA TAC AAA CTT  1344
        G   Q   G   C   V   E   I   G   S   Q   R   Y   K   L

1345  GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC ATG CTT AAA  1386
        G   V   R   L   Y   Y   R   V   M   E   S   M   L   K

1387  TCA GAA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA CTT  1428
        S   E   E   E   R   L   S   I   Q   N   F   S   K   L

1429  CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT  1470
        L   N   D   N   I   F   H   M   S   L   L   A   C   A

1471  CTT GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG  1512
        L   E   V   V   M   A   T   Y   S   R   S   T   S   Q

1513  AAT CTT GAT TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG  1554
        N   L   D   S   G   T   D   L   S   F   P   W   I   L

1555  AAT GTG CTT AAT TTA AAA GCC TTT GAT TTT TAC AAA GTG ATC  1596
        N   V   L   N   L   K   A   F   D   F   Y   K   V   I

1597  GAA AGT TTT ATC AAA GCA GAA GGC AAC TTG ACA AGA GAA ATG  1638
        E   S   F   I   K   A   E   G   N   L   T   R   E   M

1639  ATA AAA CAT TTA GAA CGA TGT GAA CAT CGA ATC ATG GAA TCC  1680
        I   K   H   L   E   R   L   E   H   R   I   M   E   S

1681  CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT CTT ATT AAA  1722
        L   A   W   L   S   D   S   P   L   F   D   L   I   K

1723  CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA TCT  1764
        Q   S   K   D   R   E   G   P   T   D   H   L   E   S

1765  GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA  1806
        A   C   P   L   N   L   P   L   Q   N   N   H   T   A

1807  GCA GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA  1848
        A   D   M   Y   L   S   P   V   R   S   P   K   K   K

1849  GGT TCA ACT ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA  1890
        G   S   T   T   R   V   N   S   T   A   N   A   E   T

1891  CAA GCA ACC TCA GCC TTC CAG ACC CAG AAG CCA TTG AAA TCT  1932
        Q   A   T   S   A   F   Q   T   Q   K   P   L   K   S

1933  ACC TCT CTT TCA CTG TTT TAT AAA AAA GTG TAT CGG CTA GCC  1974
        T   S   L   S   L   F   Y   K   K   V   Y   R   L   A

1975  TAT CTC CGG CTA AAT ACA CTT TGT GAA CGC CTT CTG TCT GAG  2016
        Y   L   R   L   N   T   L   C   E   R   L   L   S   E

2017  CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT TTC CAG CAC  2058
        H   P   E   L   E   H   I   I   W   T   L   F   Q   H

2059  ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT TTG  2100
        T   L   Q   N   E   Y   E   L   M   R   D   R   H   L

2101  GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG  2142
        D   Q   I   M   M   C   S   M   Y   G   I   C   K   V

2143  AAG AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC  2184
        K   N   I   D   L   K   F   K   I   I   V   T   A   Y
```

FIG. 5-3

```
2185  AAG GAT CTT CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT   2226
        K   D   L   P   H   A   V   Q   E   T   F   K   R   V

2227  TTG ATC AAA GAA GAG GAG TAT GAT TCT ATT ATA GTA TTC TAT   2268
        L   I   K   E   E   E   Y   D   S   I   I   V   F   Y

2269  AAC TCG GTC TTC ATG CAG AGA CTG AAA ACA AAT ATT TTG CAG   2310
        N   S   V   F   M   Q   R   L   K   T   N   I   L   Q

2311  TAT GCT TCC ACC AGG CCC CCT ACC TTG TCA CCA ATA CCT CAC   2352
        Y   A   S   T   R   P   P   T   L   S   P   I   P   H

2353  ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA CCC TTA CGG   2394
        I   P   R   S   P   Y   K   F   P   S   S   P   L   R

2395  ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT CCA   2436
        I   P   G   G   N   I   Y   I   S   P   L   K   S   P

2437  TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT   2478
        Y   K   I   S   E   G   L   P   T   P   T   K   M   T

2479  CCA AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG   2520
        P   R   S   R   I   L   V   S   I   G   E   S   F   G

2521  ACT TCT GAG AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC   2562
        T   S   E   K   F   Q   K   I   N   Q   M   V   C   N

2563  AGC GAC CGT GTG CTC AAA AGA AGT GCT GAA GGA AGC AAC CCT   2604
        S   D   R   V   L   K   R   S   A   E   G   S   N   P

2605  CCT AAA CCA CTG AAA AAA CTA CGC TTT CAT ATT GAA GGA TCA   2646
        P   K   P   L   K   K   L   R   F   D   I   E   G   S

2647  GAT GAA GCA GAT GGA AGT AAA CAT CTC CCA GGA GAG TCC AAA   2688
        D   E   A   D   G   S   K   H   L   P   G   E   S   K

2689  TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT CGA ACA CGA   2730
        F   Q   Q   K   L   A   E   M   T   S   T   R   T   R

2731  ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA AAC   2772
        M   Q   K   Q   K   M   N   D   S   M   D   T   S   N

2773  AAG GAA GAG AAA      2784
        K   E   E   K
```

```
TGAGGATCTCAGGACCTTGGTGGACACTGTGTACACCTCTGGATTCATTGTCTCTCACAGATGTGACTGTATAACTTTCC  2864
CAGGTTCTGTTTATGGCCACATTTAATATCTTCAGCTCTTTTTGTGGATATAAAATGTGCAGATGCAATTGTTTGGGTGA  2944
TTCCTAAGCCACTTGAAATGTTAGTCATTGTTATTTATACAAGATTGAAAATCTTGTGTAAATCCTGCCATTTAAAAAGT  3024
TGTAGCAGATTGTTTCCTCTTCCAAAGTAAAATTGCTGTGCTTTATGGATAGTAAGAATGGCCCTAGAGTGGGAGTCCTG  3104
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FIG. 6-1

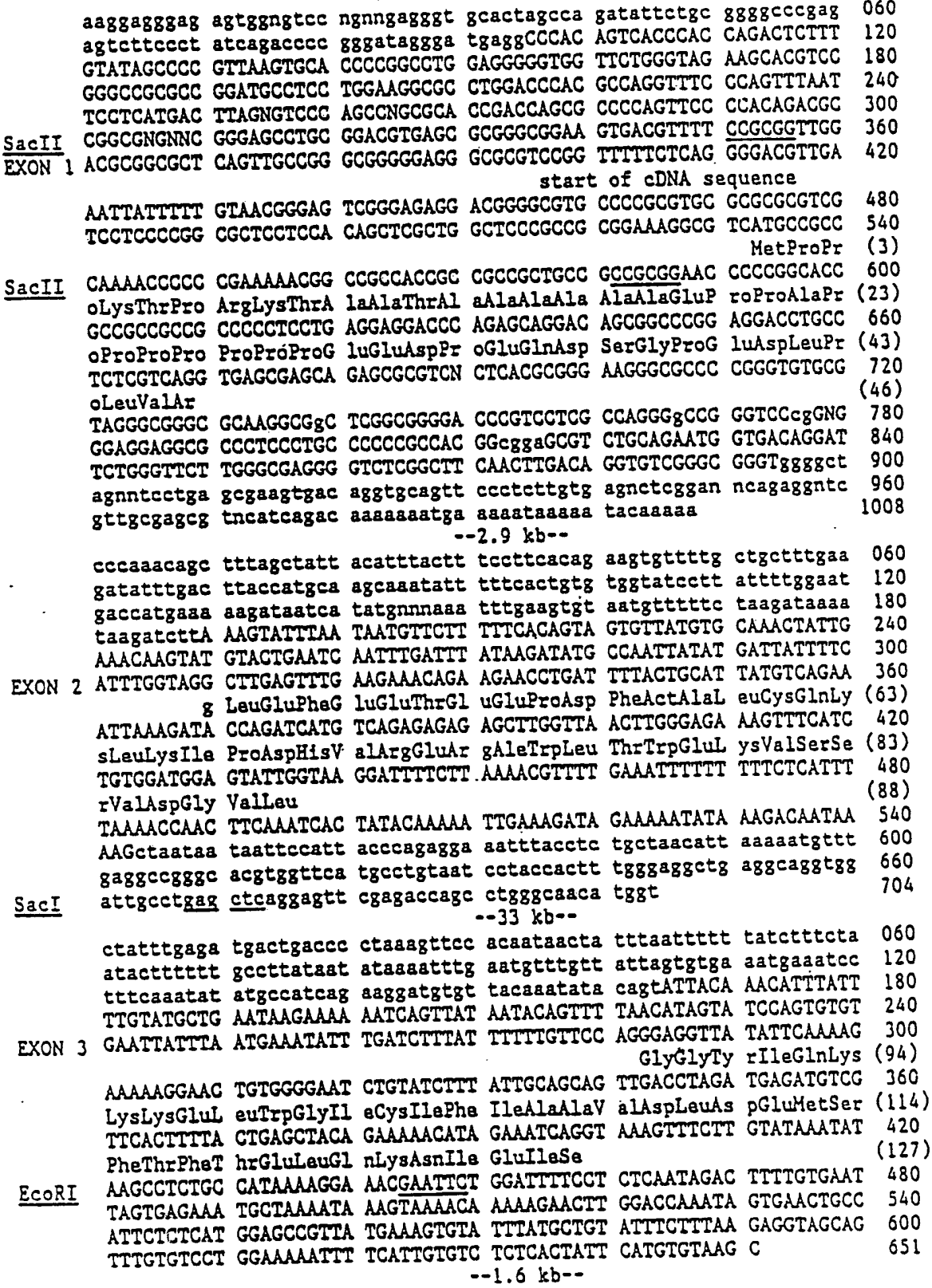
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FIG. 6-2

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EXON 4TTCTTTCCTT TGTAGTGTCC ATAAATTCTT TAACTTACTA AAAGAAATTG ATACCAGTAC  240
                  rValH isLysPhePh eAsnLeuLeu LysGluIleA spThrSerTh (142)
      CAAAGTTGAT AATGCTATGT CAAGACTGTT GAAGAAGTAT GATGTATTGT TTGCACTCTT  300
      rLysValAsp AsnAlaMetS erArgLeuLe uLysLysTyr AspValLeuP heAlaLeuPh (162)
      CAGCAAATTG GAAAGGTAAA GTAAACATTT TATTAGGGTT ACACTCTGAT TTTTTATGTC  360
      eSerLysLeu GluAr                                                  (167)
      ATTGTTCACA ATTAGATTCT GGGAATTATT TAACACATTT AGTAAAGTTA GTAAGTATTA  420
      ATTCTTAgac ttgtcccttt taatgttagc tcattaattc ttagctttct tatttatcca  480
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EXON 5TGACTTCTAA ATTACGAAAA AATGTTAAAA AGTCATAATG TTTTTCTTTT CAGGACATGT  300
                                                        . gThrCys (169)
      GAACTTATAT ATTTGACACA ACCCAGCAGT TCGTAAGTAG TTCACAGAAT GTTATTTTTC  360
      GluLeuIleT yrLeuThrGl nProSerSer Se                               (180)
      ACTTAAAAAA AAAGATTTTT ATGGAATAAT CTCAAACATC TTGATAGTTA GGGTTAGTTT  420
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EXON 6TTTCTGCTTT CTATTTGTTT AATAGGATAT CTACTGAAAT AAATTCTGCA TTGGTGCTAA  180
                              rIleS erThrGluIl eAsnSerAla LeuValLeuL (192)
      AAGTTTCTTG GATCACATTT TTATTAGCTA AAGGTAAGTT CATTATATTT ATTAAATGCT  240
      ysValSerTr pIleThrPhe LeuLeuAlaL ysG                              (203)
      AATATTTCAA ATGTAATAAT TAAATTGGCA TTCCTTTGGA CTAAATTCCC CAATTTTTAT  300
      TGAGTAATGT ACTCCTccct cattctctgc ttggcttatt aactgttagc aagttcctat  360
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      TTTCTCTCAT ACAAAGATCT GAATCTCTAA CTTTCTTTAA AAATGTACAT TTTTTTTTCA  300
EXON 7GGGGAAGTAT TACAAATGGA AGATGATCTG GTGATTTCAT TTCAGTTAAT GCTATGTGTC  360
       lyGluValL euGlnMetGl uAspAspLeu ValIleSerP heGlnLeuMe tLeuCysVal (222)
      CTTGACTATT TTATTAAACT CTCACCTCCC ATGTTGCTCA AAGAACCATA TAGTAAGTAT  420
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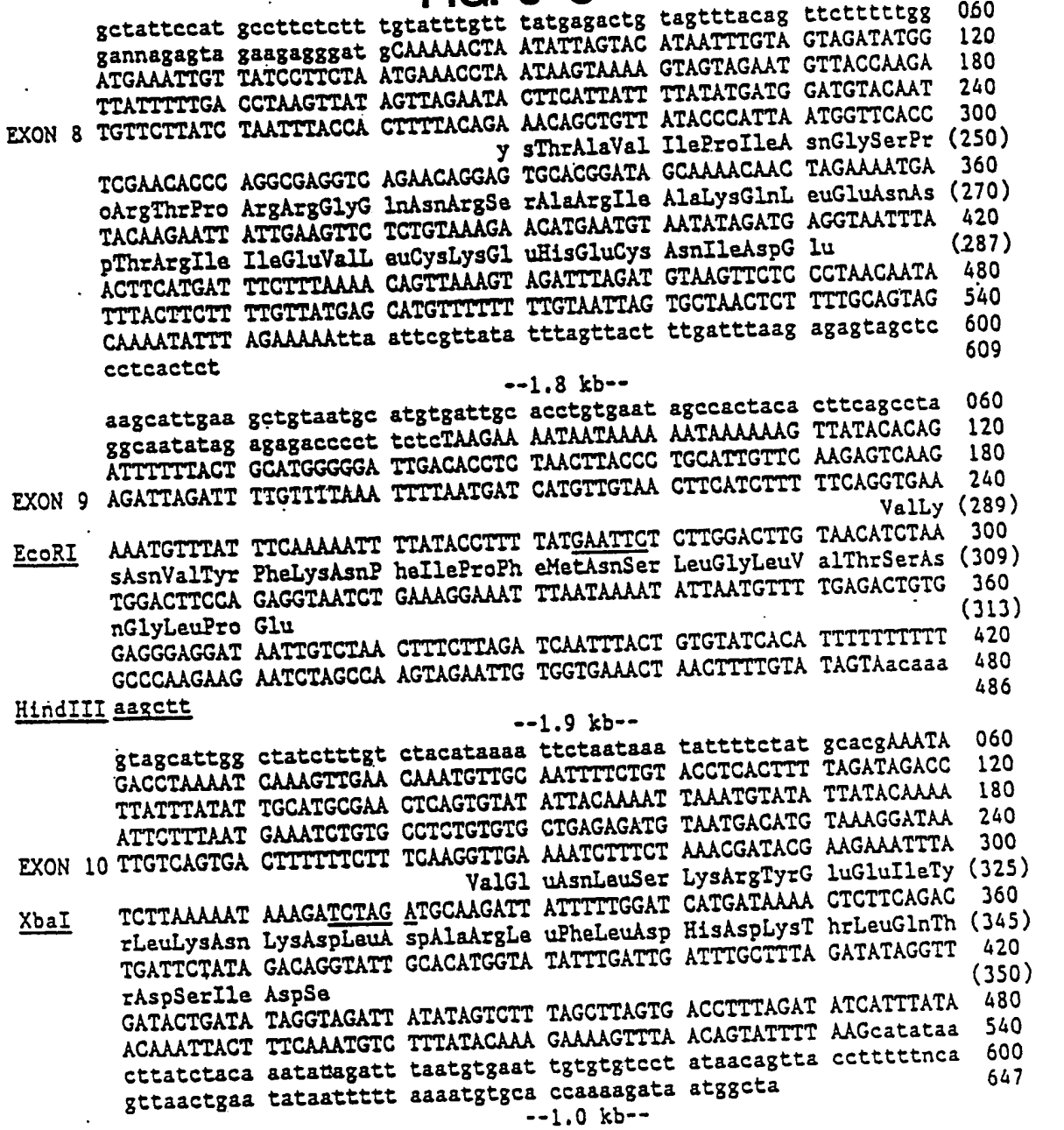
FIG. 6-3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EXON 9 AGATTAGATT TTGTTTTAAA TTTTAATGAT CATGTTGTAA CTTCATCTTT TTCAGGTGAA 240
ValLy (289)
EcoRI AAATGTTTAT TTCAAAAATT TTATACCTTT TATGAATTCT CTTGGACTTG TAACATCTAA 300
sAsnValTyr PheLysAsnP heIleProPh eMetAsnSer LeuGlyLeuV alThrSerAs (309)
TGGACTTCCA GAGGTAATCT GAAAGGAAAT TTAATAAAAT ATTAATGTTT TGAGACTGTG 360
(313)
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EXON 10 TTGTCAGTGA CTTTTTTCTT TCAAGGTTGA AAATCTTTCT AAACGATACG AAGAAATTTA 300
ValGl uAsnLeuSer LysArgTyrG luGluIleTy (325)
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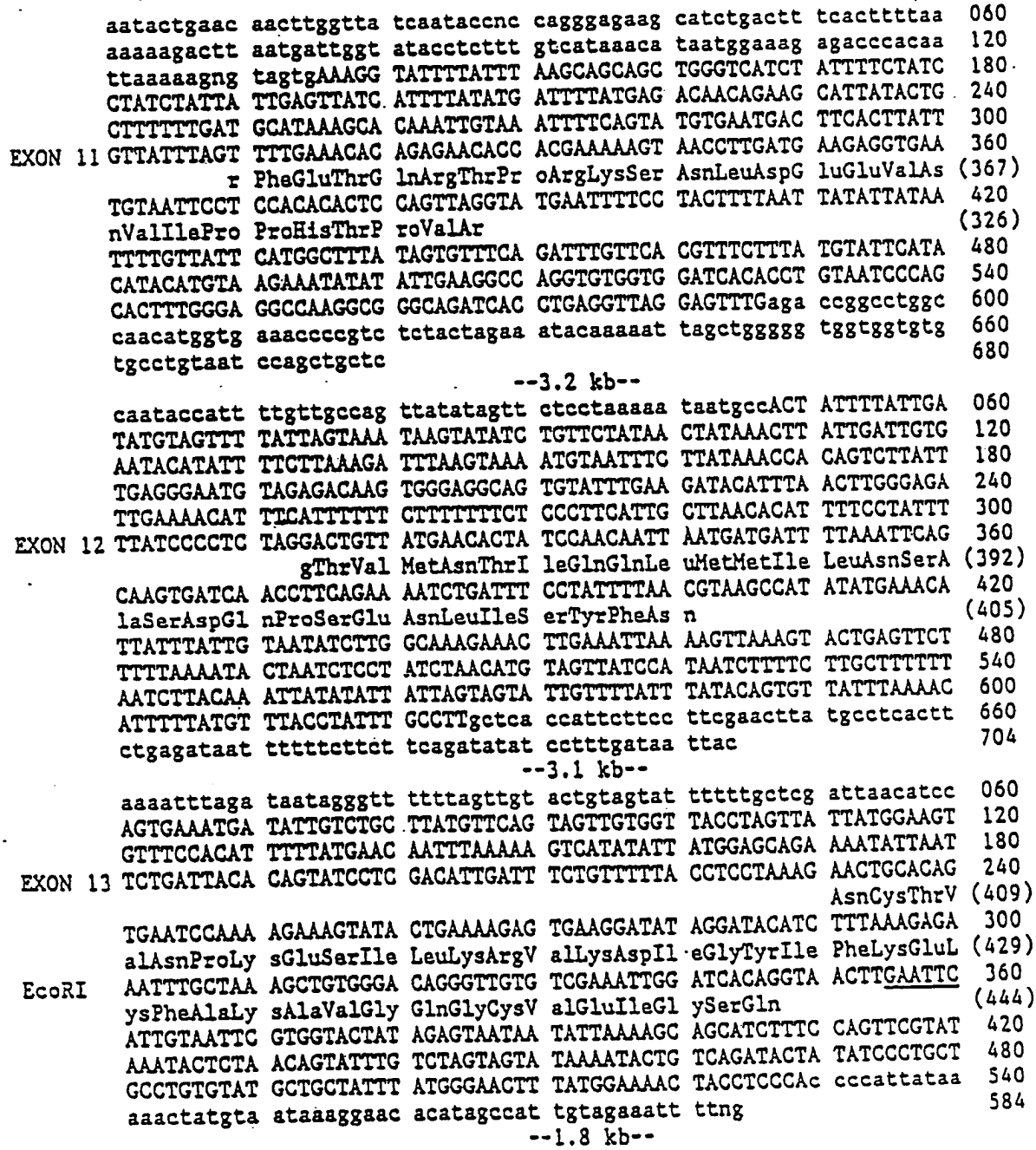
FIG. 6-4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nValIlePro ProHisThrP roValAr (326)
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CATACATGTA AGAAATATAT ATTGAAGGCC AGGTGTGGTG GATCACACCT GTAATCCCAG 540
CACTTTGGGA GGCCAAGGCG GGCAGATCAC GTGAGGTTAG GAGTTTGaga ccggcctggc 600
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tgcctgtaat ccagctgctc 680
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TATGTAGTTT TATTAGTAAA TAAGTATATC TGTTCTATAA CTATAAACTT ATTGATTGTG 120
AATACATATT TTCTTAAAGA TTTAAGTAAA ATGTAATTTC TTATAAACCA CAGTCTTATT 180
TGAGGGAATG TAGAGACAAG TGGGAGGCAG TGTATTTGAA GATACATTTA ACTTGGGAGA 240
TTGAAAACAT TTCATTTTTT CTTTTTTTCT CCCTTCATTG CTTAACACAT TTTCCTATTT 300
EXON 12 TTATCCCCTC TAGGACTGTT ATGAACACTA TCCAACAATT AATGATGATT TTAAATTCAG 360
gThrVal MetAsnThrI leGlnGlnLe uMetMetIle LeuAsnSerA (392)
CAAGTGATCA ACCTTCAGAA AATCTGATTT CCTATTTTAA CGTAAGCCAT ATATGAAACA 420
laSerAspGl nProSerGlu AsnLeuIleS erTyrPheAs n (405)
TTATTTATTG TAATATCTTG GCAAAGAAAC TTGAAATTAA AAGTTAAAGT ACTGAGTTCT 480
TTTTAAAATA CTAATCTCCT ATCTAACATG TAGTTATCCA TAATCTTTTC TTGCTTTTTT 540
AATCTTACAA ATTATATATT ATTAGTAGTA TTGTTTTATT TATACAGTGT TATTTAAAAC 600
ATTTTTATGT TTACCTATTT GCCTTgctca ccattcttcc ttcgaactta tgcctcactt 660
ctgagataat ttttttcttct tcagatatat cctttgataa ttac 704
--3.1 kb--
aaaatttaga taatagggtt ttttagttgt actgtagtat tttttgctcg attaacatcc 060
AGTGAAATGA TATTGTCTGC TTATGTTCAG TAGTTGTGGT TACCTAGTTA TTATGGAAGT 120
GTTTCCACAT TTTTATGAAC AATTTAAAAA GTCATATATT ATGGAGCAGA AAATATTAAT 180
EXON 13 TCTGATTACA CAGTATCCTC GACATTGATT TCTGTTTTTA CCTCCTAAAG AACTGCACAG 240
AsnCysThrV (409)
TGAATCCAAA AGAAAGTATA CTGAAAAGAG TGAAGGATAT AGGATACATC TTTAAAGAGA 300
alAsnProLy sGluSerIle LeuLysArgV alLysAspIl eGlyTyrIle PheLysGluL (429)
EcoRI AATTTGCTAA AGCTGTGGGA CAGGGTTGTG TCGAAATTGG ATCACAGGTA ACTTGAATTC 360
ysPheAlaLy sAlaValGly GlnGlyCysV alGluIleGl ySerGln (444)
ATTGTAATTC GTGGTACTAT AGAGTAATAA TATTAAAAGC AGCATCTTTC CAGTTCGTAT 420
AAATACTCTA ACAGTATTTG TCTAGTAGTA TAAAATACTG TCAGATACTA TATCCCTGCT 480
GCCTGTGTAT GCTGCTATTT ATGGGAACTT TATGGAAAAC TACCTCCCAc cccattataa 540
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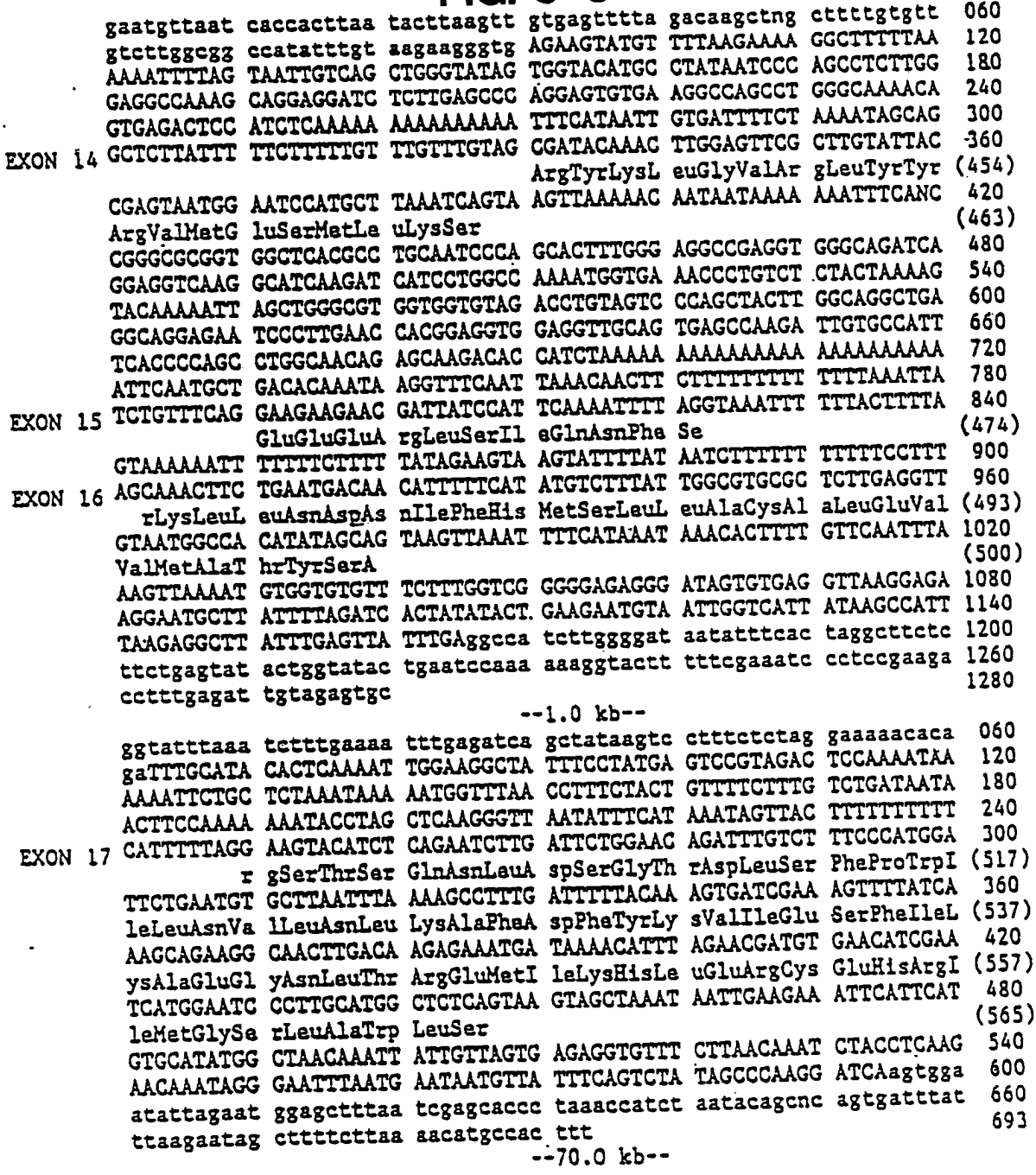
FIG. 6-5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GAGGCCAAAG CAGGAGGATC TCTTGAGCCC AGGAGTGTGA AGGCCAGCCT GGGCAAAACA 240
GTGAGACTCC ATCTCAAAAA AAAAAAAAAA TTTCATAATT GTGATTTTCT AAAATAGCAG 300
EXON 14 GCTCTTATTT TTCTTTTTGT TTGTTTGTAG CGATACAAAC TTGGAGTTCG CTTGTATTAC 360
ArgTyrLysL euGlyValAr gLeuTyrTyr (454)
CGAGTAATGG AATCCATGCT TAAATCAGTA AGTTAAAAAC AATAATAAAA AAATTTCANC 420
(463)
ArgValMetG luSerMetLe uLysSer
CGGGCGCGGT GGCTCACGCC TGCAATCCCA GCACTTTGGG AGGCCGAGGT GGGCAGATCA 480
GGAGGTCAAG GCATCAAGAT CATCCTGGCC AAAATGGTGA AACCCTGTCT CTACTAAAAG 540
TACAAAAAAT AGCTGGGCGT GGTGGTGTAG ACCTGTAGTC CCAGCTACTT GGCAGGCTGA 600
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TCACCCCAGC CTGGCAACAG AGCAAGACAC CATCTAAAAA AAAAAAAAAA AAAAAAAAAA 720
ATTCAATGCT GACACAAATA AGGTTTCAAT TAAACAACTT CTTTTTTTTT TTTTAAATTA 780
EXON 15 TCTGTTTCAG GAAGAAGAAC GATTATCCAT TCAAAATTTT AGGTAAATTT TTTACTTTTA 840
GluGluGluA rgLeuSerIl eGlnAsnPhe Se (474)
GTAAAAAATT TTTTTCTTTT TATAGAAGTA AGTATTTTAT AATCTTTTTT TTTTTCCTTT 900
EXON 16 AGCAAACTTC TGAATGACAA CATTTTTCAT ATGTCTTTAT TGGCGTGCGC TCTTGAGGTT 960
rLysLeuL euAsnAspAs nIlePheHis MetSerLeuL euAlaCysAl aLeuGluVal (493)
GTAATGGCCA CATATAGCAG TAAGTTAAAT TTTCATAAAT AAACACTTTT GTTCAATTTA 1020
ValMetAlaT hrTyrSerA (500)
AAGTTAAAAT GTGGTGTGTT TCTTTGGTCG GGGGAGAGGG ATAGTGTGAG GTTAAGGAGA 1080
AGGAATGCTT ATTTTAGATC ACTATATACT. GAAGAATGTA ATTGGTCATT ATAAGCCATT 1140
TAAGAGGCTT ATTTGAGTTA TTTGAggcca tcttggggat aatatttcac taggcttctc 1200
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cctttgagat tgtagagtgc 1280
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AAAATTCTGC TCTAAATAAA AATGGTTTAA CCTTTCTACT GTTTTCTTTG TCTGATAATA 180
ACTTCCAAAA AAATACCTAG CTCAAGGGTT AATATTTCAT AAATAGTTAC TTTTTTTTTT 240
EXON 17 CATTTTTAGG AAGTACATCT CAGAATCTTG ATTCTGGAAC AGATTTGTCT TTCCCATGGA 300
r gSerThrSer GlnAsnLeuA spSerGlyTh rAspLeuSer PheProTrpI (517)
TTCTGAATGT GCTTAATTTA AAAGCCTTTG ATTTTTACAA AGTGATCGAA AGTTTTATCA 360
leLeuAsnVa lLeuAsnLeu LysAlaPheA spPheTyrLy sValIleGlu SerPheIleL (537)
AAGCAGAAGG CAACTTGACA AGAGAAATGA TAAAACATTT AGAACGATGT GAACATCGAA 420
ysAlaGluGl yAsnLeuThr ArgGluMetI leLysHisLe uGluArgCys GluHisArgI (557)
TCATGGAATC CCTTGCATGG CTCTCAGTAA GTAGCTAAAT AATTGAAGAA ATTCATTCAT 480
(565)
leMetGlySe rLeuAlaTrp LeuSer
GTGCATATGG CTAACAAATT ATTGTTAGTG AGAGGTGTTT CTTAACAAAT CTACCTCAAG 540
AACAAATAGG GAATTTAATG AATAATGTTA TTTCAGTCTA TAGCCCAAGG ATCAagtgga 600
atattagaat ggagctttaa tcgagcaccc taaaccatct aatacagcnc agtgatttat 660
ttaagaatag cttttcttaa aacatgccac ttt 693
--70.0 kb--

FIG. 6-6

```
          attttctaat ataagcgttg aaggttatac atttttctac ttttttgtgt gtgggaagta  060
          caaaaattgt CAATTGGGAA TTTCGAAGTA GAGAAAAATA TTTCATTCTG ACTTTTAAAT  120
          TGCCACTGTC AATTGTGCCT AAAATTCATA GTACTTACCA TGTCAAACAA TATGATTTTG  180
          ATATGTACCT GGGAAAATTA TGCTTACTAA TGTGGTTTTA ATTTCATCAT GTTTCATATA  240
EXON 18   GGATTCACCT TTATTTGATC TTATTAAACA ATCAAAGGAC CGAGAAGGAC CAACTGATCA  300
           AspSerPro LeuPheAspL euIleLysGl nSerLysAsp ArgGluGlyP roThrAspHi (585)
          CCTTGAATCT GCTTGTCGTC TTAATCTTCC TCTCCAGAAT AATCACACTG CAGCAGATAT  360
          sLeuGluSer AlaCysProL euAsnLeuPr oLeuGlnAsn AsnHisThrA laAlaAspMe (605)
          GTAAGCAAAA TATATGTTAT GTTGACCATC AAACTGCAAA TAGATTTTAA GCATAAGTGC  420
          AATGTAACAT TCTATAAAGA AAGTGTAGGG AATAGAATTT TGAATAAGAA TAGTTTCTGT  480
          TTTTAAGAAA TTAGTAATAA AAGGTACATg acccaaataa agtcatataa aagagtacag  540
          agtgctactg aatcacctag gatttgcata atgagagcag ttttcatgg              589
                                        --3.0 kb--
          tgtttttaag ctggaatcac cttatggtct caataccact ataattatta aaattgTACA  060
          TTATACATAT ATAGCTATTT TTTTCTAATA AGGCAGTAAT CCCCAGGAAA AGCCATTTAT  120
          TAAAATAGAA TTAGATATGA TGATGACAAG CAGTTTTCGT ATTAATATAT CTTTCCCAGC  180
          TTGCATTTAA ATAGTCTGCT ATAATACCAA TTAAATAGAC AAGATGTATC TGGGTGTACA  240
          ACCTTGAAGT GTATGTATAA TCTGTGATTC TTAGCCAACT TGAAATGAAG ACTTTTCCTT  300
EXON 19   TAAATATATC TAGGTATCTT TCTCCTGTAA GATCTCCAAA GAAAAAAGGT TCAACTACGC  360
                    tTyrLeu SerProValA rgSerProLy sLysLysGly SerThrThrA (621)
          GTGTAAATTC TACTGCAAAT GCAGAGACAC AAGCAACCTC AGCCTTCCAG ACCCAGAAGC  420
          rgValAsnSe rThrAlaAsn AlaGluThrG lnAlaThrSe rAlaPheGln ThrGlnLysP (641)
          CATTGAAATC TACCTCTCTT TCACTGTTTT ATAAAAAAGG TTAGTAGATG ATTATTTTCA  480
          roLeuLysSe rThrSerLeu SerLeuPheT yrLysLysV                         (654)
          AGAGCATGGA CTCTGAAACT AGGCTGACTG GGTTCAAATC ATGTTTCTTC TACTTTCTAG  540
          GTACATTACT GGGCAAGTCA CTTAATATCT CTGTGTCTCA GTTTCCTcat ctataaaatg  600
          gaaatgataa tgttgcgaga tctttcttga ctattcagag tcgttttctg              650
                                        --2.8 kb--
          aaggaaaatc catgcccent cgggacatgc ctgnccteig catttcttca tctgtatccc  060
          ttgtaatatg cctcataata aaccagtaaa catGTTTCTC TGGGGGAAAG AAAAGAGTGG  120
          TAGAAAAGAG GTTTCTGTTA AAATGCTACT TAACAGCATT ATAATTAGTG TAATTTCATG  180
          ATTTGAAAAA AATCTACTTG TAATTCAAAA TGAACAGTAA AAATGACTAA TTTTTCTTAT  240
EXON 20   TCCCACAGTG TATCGGCTAG CCTATCTCCG GCTAAATACA CTTTGTGAAC GCCTTCTGTC  300
                al TyrArgLeuA laTyrLeuAr gLeuAsnThr LeuCysGluA rgLeuLeuSe (671)
          TGAGCACCCA GAATTAGAAC ATATCATCTG GACCCTTTTC CAGCACACCC TGCAGAATGA  360
          rGluHisPro GluLeuGluH isIleIleTr pThrLeuPhe GlnHisThrL euGlnAsnGl (691)
          GTATGAACTC ATGAGAGACA GGCATTTGGA CCAAGTAAGA AAATCAAGCA CTTCACCTTC  420
          uTyrGluLeu MetArgAspA rgHisLeuAs pGln                             (702)
          TCTCCTCCCT ACTTACTTGT TAACTGATTT CTTTCTTTCT TTCTTTCTTT CTTTCTTTCT  480
          TTCTTTCTTT CTTTCTTTCT TTCTTTTCTT TCTTTTCTTT GTTTCTTTCT TTCCTTTTTT  540
          TTTTTTTGAG ATAGAGTCTC ACTCTGTTAC CCAGGCTGGA GTGCAGTGGC GCAATCTCGG  600
          CTCACTGCAA CCTCCGCCTC CCAGGTCAAG TGATTCTCCT GCCTCAGCCT ccnaggagct  660
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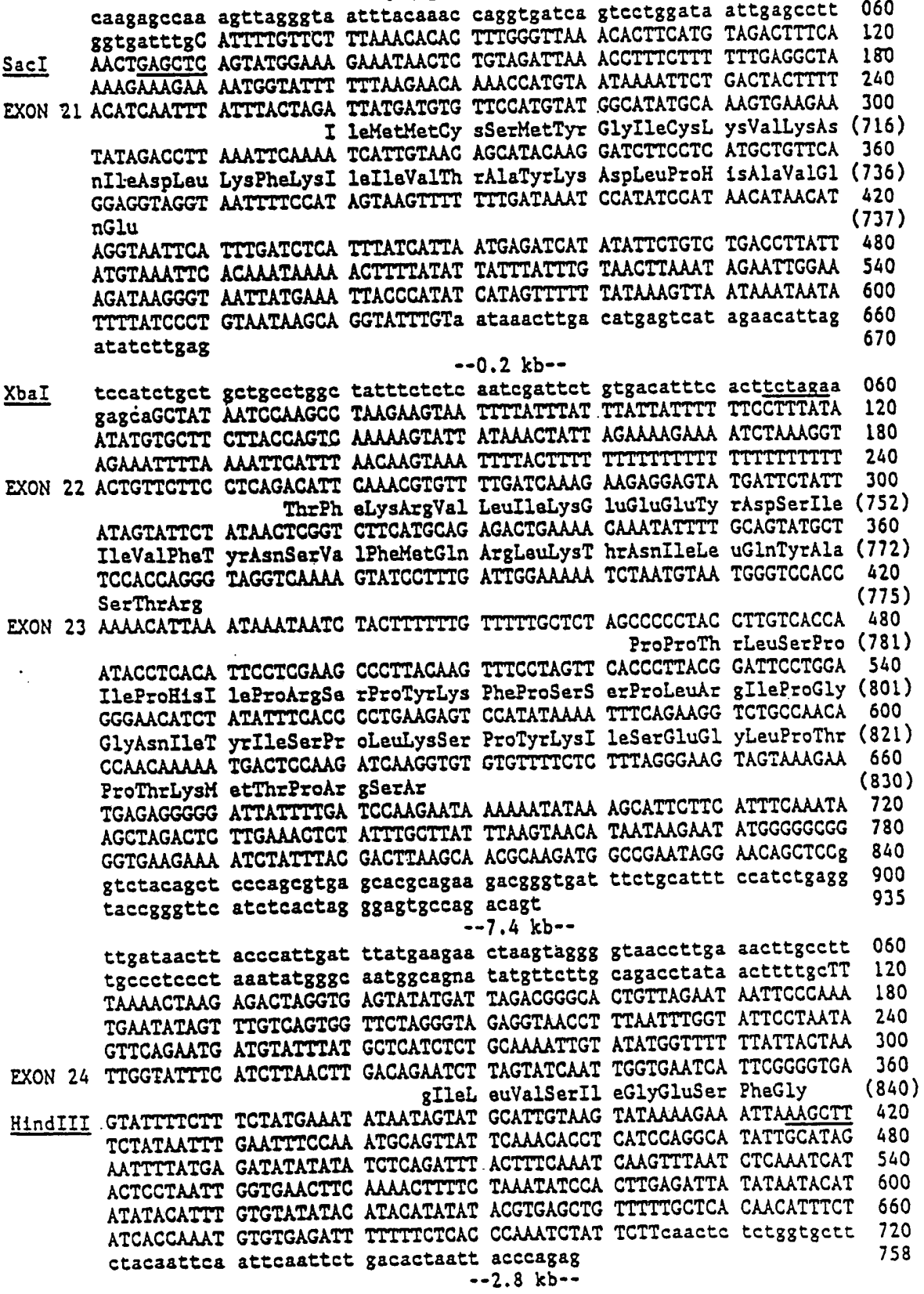
FIG. 6-7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SacI AACTGAGCTC AGTATGGAAA GAAATAACTC TGTAGATTAA ACCTTTCTTT TTTGAGGCTA 180
AAAGAAAGAA AATGGTATTT TTTAAGAACA AAACCATGTA ATAAAATTCT GACTACTTTT 240
EXON 21 ACATCAATTT ATTTACTAGA TTATGATGTG TTCCATGTAT GGCATATGCA AAGTGAAGAA 300
I leMetMetCy sSerMetTyr GlyIleCysL ysValLysAs (716)
TATAGACCTT AAATTCAAAA TCATTGTAAC AGCATACAAG GATCTTCCTC ATGCTGTTCA 360
nIleAspLeu LysPheLysI leIleValTh rAlaTyrLys AspLeuProH isAlaValGl (736)
GGAGGTAGGT AATTTTCCAT AGTAAGTTTT TTTGATAAAT CCATATCCAT AACATAACAT 420
nGlu (737)
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ATGTAAATTC ACAAATAAAA ACTTTTATAT TATTTATTTG TAACTTAAAT AGAATTGGAA 540
AGATAAGGGT AATTATGAAA TTACCCATAT CATAGTTTTT TATAAAGTTA ATAAATAATA 600
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atatcttgag 670
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AGAAATTTTA AAATTCATTT AACAAGTAAA TTTTACTTTT TTTTTTTTTT TTTTTTTTTT 240
EXON 22 ACTGTTCTTC CTCAGACATT CAAACGTGTT TTGATCAAAG AAGAGGAGTA TGATTCTATT 300
ThrPh eLysArgVal LeuIleLysG luGluGluTy rAspSerIle (752)
ATAGTATTCT ATAACTCGGT CTTCATGCAG AGACTGAAAA CAAATATTTT GCAGTATGCT 360
IleValPheT yrAsnSerVa lPheMetGln ArgLeuLysT hrAsnIleLe uGlnTyrAla (772)
TCCACCAGGG TAGGTCAAAA GTATCCTTTG ATTGGAAAAA TCTAATGTAA TGGGTCCACC 420
SerThrArg (775)
EXON 23 AAAACATTAA ATAAATAATC TACTTTTTTG TTTTTGCTCT AGCCCCCTAC CTTGTCACCA 480
ProProTh rLeuSerPro (781)
ATACCTCACA TTCCTCGAAG CCCTTACAAG TTTCCTAGTT CACCCTTACG GATTCCTGGA 540
IleProHisI leProArgSe rProTyrLys PheProSerS erProLeuAr gIleProGly (801)
GGGAACATCT ATATTTCACC CCTGAAGAGT CCATATAAAA TTTCAGAAGG TCTGCCAACA 600
GlyAsnIleT yrIleSerPr oLeuLysSer ProTyrLysI leSerGluGl yLeuProThr (821)
CCAACAAAAA TGACTCCAAG ATCAAGGTGT GTGTTTTCTC TTTAGGGAAG TAGTAAAGAA 660
ProThrLysM etThrProAr gSerAr (830)
TGAGAGGGGG ATTATTTTGA TCCAAGAATA AAAAATATAA AGCATTCTTC ATTTCAAATA 720
AGCTAGACTC TTGAAACTCT ATTTGCTTAT TTAAGTAACA TAATAAGAAT ATGGGGGCGG 780
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TGAATATAGT TTGTCAGTGG TTCTAGGGTA GAGGTAACCT TTAATTTGGT ATTCCTAATA 240
GTTCAGAATG ATGTATTTAT GCTCATCTCT GCAAAATTGT ATATGGTTTT TTATTACTAA 300
EXON 24 TTGGTATTTC ATCTTAACTT GACAGAATCT TAGTATCAAT TGGTGAATCA TTCGGGGTGA 360
gIleL euValSerIl eGlyGluSer PheGly (840)
HindIII GTATTTTCTT TCTATGAAAT ATAATAGTAT GCATTGTAAG TATAAAAGAA ATTAAAGCTT 420
TCTATAATTT GAATTTCCAA ATGCAGTTAT TCAAACACCT CATCCAGGCA TATTGCATAG 480
AATTTTATGA GATATATATA TCTCAGATTT ACTTTCAAAT CAAGTTTAAT CTCAAATCAT 540
ACTCCTAATT GGTGAACTTC AAAACTTTTC TAAATATCCA CTTGAGATTA TATAATACAT 600
ATATACATTT GTGTATATAC ATACATATAT ACGTGAGCTG TTTTTGCTCA CAACATTTCT 660
ATCACCAAAT GTGTGAGATT TTTTTCTCAC CCAAATCTAT TCTTcaactc tctggtgctt 720
ctacaattca attcaattct gacactaatt acccagag 758
--2.8 kb--

FIG. 6-8

```
        ggggatggaa ttaggtagtt attctgattt ttAGATTTTT CATATCTTTT ATTTGGTCCA  060
        ATGAAGCAGA AAATTTAAAT GAAGTTATTA CCTTTGCCTG ATTTTTGACA CACCTCAAAC  120
EcoRI   TATAACTTGA GGTTGCTAAC TATGAAACAC TGGCATTTAA TGATTTAAAG TAAAGAATTC  180
EXON 25 TGTAATTTGT AGACTTCTGA GAAGTTCCAG AAAATAAATC AGATGGTATG TAACAGCGAC  240
                  ThrSerGl uLysPheGln LysIleAsnG lnMetValCy sAsnSerAsp (856)
        CGTGTGCTCA AAAGAAGTGC TGAAGGAAGC AACCCTCCTA AACCACTGAA AAAACTACGC  300
        ArgValLeuL ysArgSerAl aGluGlySer AsnProProL ysProLeuLy sLysLeuArg (876)
        TTTGATATTG AAGGATCAGA TGAAGCAGAT GGAAGGTAGG AACCAGTTTT GAATGTTTTC  360
        PheAspIleG luGlySerAs pGluAlaAsp GlySe                             (888)
        CAGTAGCTGA GATGGTCATC TGGGGAATCC AGAGTCTCAG CACTGCTCCT GGGTTATACC  420
        AATTTCTTTC ATGCCAAGTT TATTTGGAAG TTGTGAGAAT GGCTCAAAAT AATAGATATG  480
        AGTGTAGTGC AAAGTTAAAA ACATCTTACA AATTGCATAC CAACATTCAG TGAAGATATC  540
        TAATAAACCC TGATCTTTTT TACAAAGGTA TTGATAAAAT TTTGTTATTC TTAACATTAA  600
        ATTTAAAAAT GTTTACTCTT GAAAAATATT AACCACTGTA TTTTGTGAGA ACCACTGAAA  660
        AAATACATAG CATCATAAAT TTGTGACATT TATGTTTTAG ATGGTTAGTT TTTAAATTTT  720
        AAAATTAAAA GCTACTCACT AAAATAATAG CATAAAGTAA GTCATCGAAA GCATCATAGT  780
        TACTGGAAAT TTGAGTTTTC CATTTATAAA TACACATGAA ATGTTTTGCA TTTTTTTAAT  840
EXON 26 CTGCAGTAAA CATCTCCCAG GAGAGTCCAA ATTTCAGCAG AAACTGGCAG AAATGAGTAA  900
              rLys HisLeuProG lyGluSerLy sPheGlnGln LysLeuAlaG luMetT      (905)
        GTACTTTTTT CACCTTGTGT AAACGAAATA AACAATTGTT TACACTGCAA GAAGTCTTTT  960
        CGTTATATAA AAGAATGTAT AATTTCTTCA GTTGGCAGGT TTGTTTATGC ATTTAAAATA 1020
        TAATTCAATC AAGGTTATTT ATCTACAAAC ATTTGTGGAT TAAATGTATG ATGTAAAATG 1080
        AAGGTCATTT TTACCCTTTC TATGATCTTT CATGCAGGAA GACTAAGAAG TGAAACATTG 1140
        CTTGACCACA TTCAAcacaa atggctacag ttagaaaata ctttagcaga actacaaaga 1200
        ggaactattt gggagtgtta gatataggga aaagttttat aaacctagca tatgtaaaac 1260
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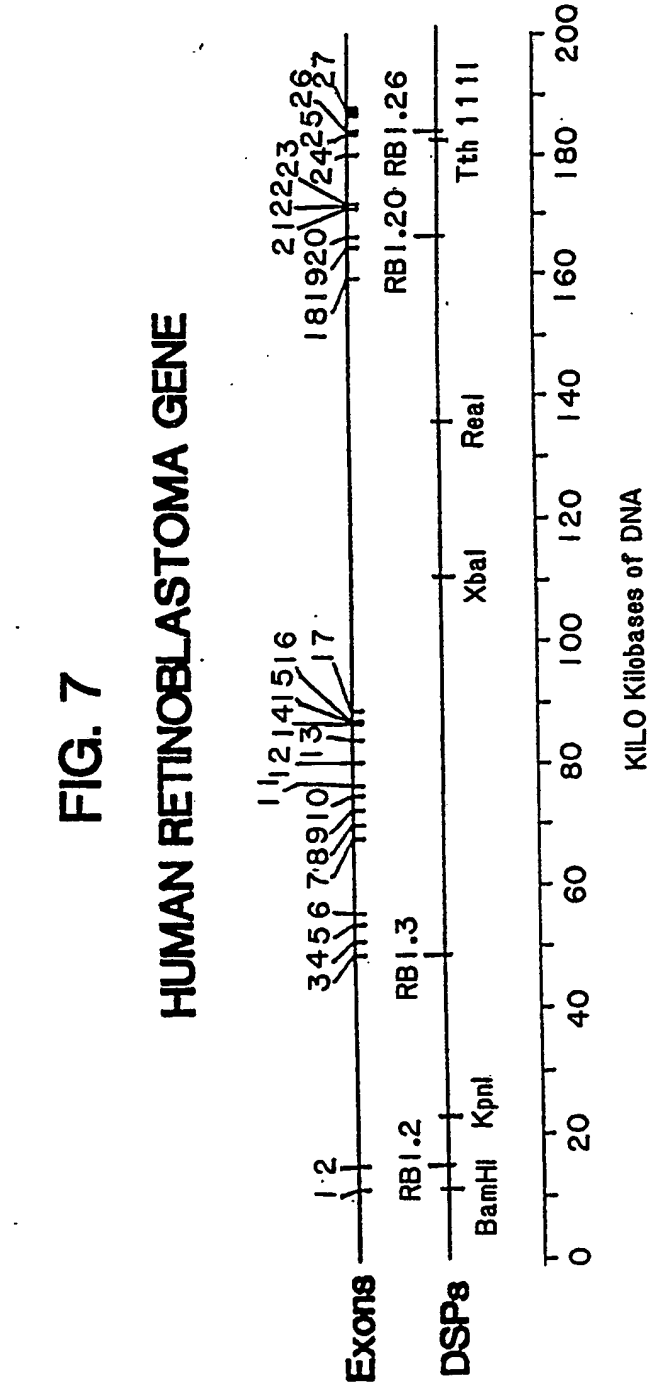
        aatgaagacc acctcttttt gcAAGGTCCT GAGCGCCATC AGTTTGACAT GAGCATAATA  120
        TATATGGCAG CCACTTGCCA ACTTACCCAG TACCATCAAT GCTGTTAACA GTTCTTCATC  180
EXON 27 CTTTTTCCAG CTTCTACTCG AACACGAATG CAAAAGCAGA AAATGAATGA TAGCATGGAT  240
                  hrSerThrAr gThrArgMet GlnLysGlnL ysMetAsnAs pSerMetAsp (921)
        ACCTCAAACA AGGAAGAGAA ATGAGGATCT CAGGACCTTG GTGGACACTG TGTACACCTC  300
        ThrSerAsnL ysGluGluLy sEND 3 untranslated region begins here       (928)
        TGGATTCATT GTCTCTCACA GATGTGACTG TATAACTTTC CCAGGTTCTG TTTATGGCCA  360
        CATTTAATAT CTTCAGCTCT TTTTGTGGAT ATAAAATGTG CAGATGCAAT TGTTTGGGTG  420
        ATTCCTAAGC CACTTGAAAT GTTAGTCATT GTTATTTATA CAAGATTGAA AATCTTGTGT  480
        AAATCCTGCC ATTTAAAAAG TTGTAGCAGA TTGTTTCCTC TTCCAAAGTA AAATTGCTGT  540
        GCTTTATGGA TAGTAAGAAT GGCCCTAGAG TGGGAGTCCT GATAACCCAG GCCTGTCTGA  600
        CTACTTTGCC TTCTTTTGTA GCATATAGGT GATGTTTGCT CTTGTTTTTA TTAATTTATA  660
        TGTATATTTT TTTAATTTAA CATGAACACC CTTAGAAAAT GTGTCCTATC TATCTTCCAA  720
        ATGCAATTTG ATTGACTGCC CATTCACCAA AATTATCCTG AACTCTTCTG CAAAAATGGA  780
        TATTATTAGA AATTAGAAAA AAATTACTAA TTTTACACAT TAGATTTTAT TTTACTATTG  840
        GAATCTGATA TACTGTGTGC TTGTTTTATA AAATTTTGCT TTTAATTAAA TAAAAGCTGG  900
        AAGCAAAGTA TAACCATATG ATACTATCAT ACTACTGAAA CAGATTTCAT ACCTCAGAAT  960
        GTAAAAGAAC TTACTGATTA TTTTCTTCAT CCAACTTATG TTTTTAAATG AGGATTATTG 1020
        ATAGTACTCT TGGTTTTTAT ACCATTCAGA TCACTGAATT TATAAAGTAC CCATCTAGTA 1080
        CTTGAAAAAG TAAAGTGTTC TGCCAGATCT TAGGTATAGA GGACCCTAAC ACAGTATATC 1140
        CCAAGTGCAC TTTCTAATGT TTCTGGGTCC TGAAGAATTA AGATACAAAT TAATTTTACT 1200
        CCATAAACAG ACTGTTAATT ATAGGAGCCT TAATTTTTTT TTCATAGAGA TTTGTCTAAT 1260
        TGCATCTCAA AATTATTCTG CCCTCCTTAA TTTGGGAAGG TTTGTGTTTT CTCTGGAATG 1320
        GTACATGTCT TCCATGTATC TTTTGAACTG GCAATTGTCT ATTTATCTTT TATTTTTTTA 1380
        AGTCAGTATG GTCTAACACT GGCATGTTCA AAGCCAGATT ATTTCTAGTC CAAAATTACA 1440
HindIII AGTAATCAAG GGTCATTATG GGTTAGGCAT TAATGTTTCT ATCTGATTTT GTGCAAAAGC 1500
```

FIG. 6-9

EXON 27 TTCAAATTAA AACAGGTGCA TTAGAAAAAG AGGCGCTTCT CGCCTCCCCT ACACCTAAAG 1560
(CONTD) 3' untranslated region (continued)
GTGTATTTAA ACTATGTTGT GTGATTAACT TATTTAGAGA TGCTGTAACT TAAAATAGGG 1620
GATATTTAAG GTAGCTTCAG CTAGCTTTTA GGAAAATCAC TTTGTCTAAC TCAGAATTAT 1680
TTTTAAAAAG AAATCTGGTC TTGTTAGAAA ACAAAATTTT ATTTTGTGCT CATTTAAGTT 1740
TCAAACTTAC TATTTTGACA GTTATTTTGA TAACAATGAC AGTAGAAAAC TTGACTCCAT 1800
TTCATCATTG TTTCTGCATG AATATCATAC AAATCAGTTA GTTTTTAGGT CAAGGGCTTA 1860
CTATTTCTGG GTCTTTTGCT ACTAAGTTCA CATTAGAATT AGTGCCAGAA TTTTAGGAAC 1920
TTCAGAGATC GTGTATTGAG ATTTCTTAAA TAATGCTTCA GATATTATTG CTTTATTGCT 1980
TTTTTGTATT GGTTAAAACT GTACATTTAA AATTGCTATG TTACTATTTT CTACAATTAA 2040
TAGTTTGTCT ATTTTAAAAT AAATTAGTTG TTAAGAGTC TTAATGGTCTG ATGTTGTGTT 2100
polyadenylation signal sequence polyadenylation site
CTTTGTATTA AGTACACTAA TGTTCTCTTT TCTGTCTAGG AGAAGATAGA TAGAAGATAA 2160
CTCTCCTAGT ATCTCATCCA TTCCTAGCCT TTAAGGGGCT CTATATGCTA GAGATTTCCA 2220
AATTTATTTC TTCAGCCCTG ATCTTTTCAC AGAGGTCAAG Gttttatag ccaacagaac 2280
tcttgattcc tactcccntc tacccaatgt ctccaaatat aaactaaaat caaataaata 2340
aaaatctttt tt 2352

FIG. 7

HUMAN RETINOBLASTOMA GENE



RB-6 FIG. 8

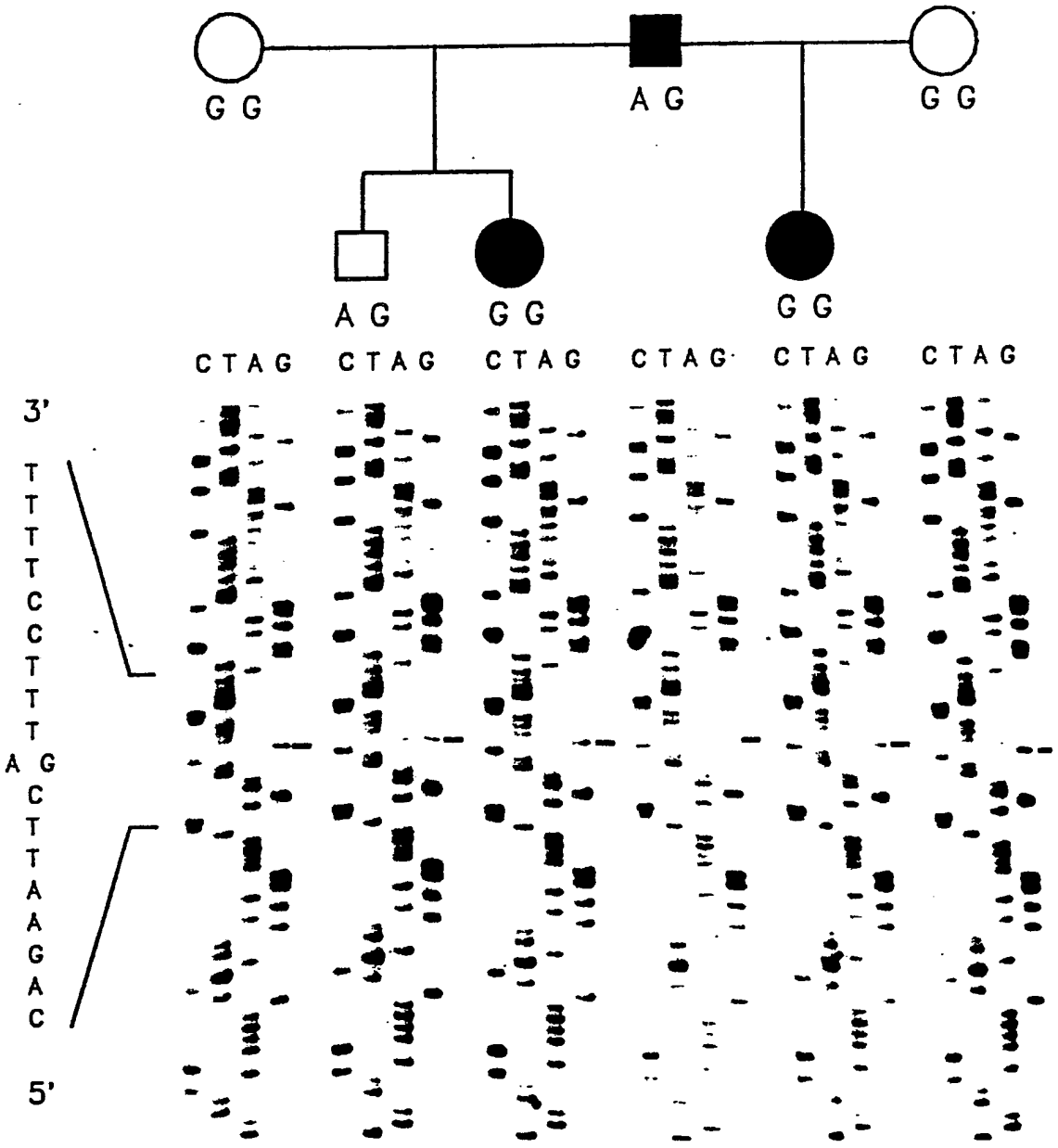
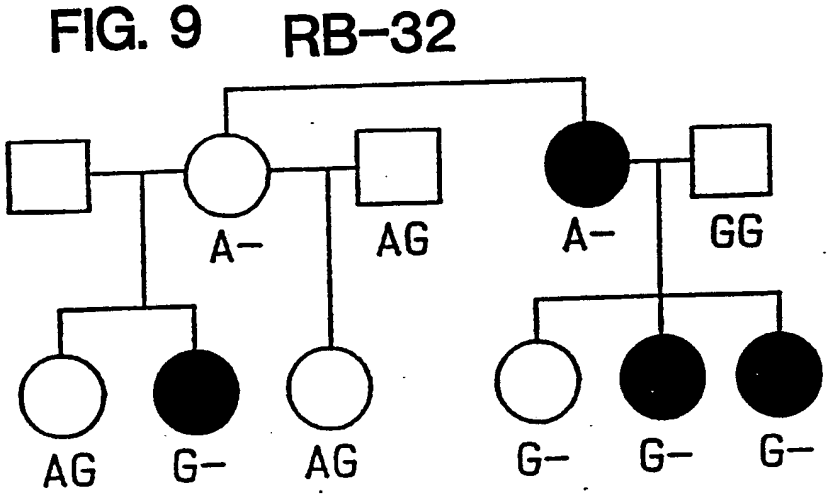

G G
A G
G G
A G
G G
G G
C T A G
C T A G
C T A G
C T A G
C T A G
C T A G
3'
T T T T C C T T T A G C T T A A G A C
5'

FIG. 9



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00293

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/68 G01N 30/00
U.S.: 435/6 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS; CAS; BIOSIS DATABASES

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	SCIENCE, Volume 235 issued 13 March 1987 (Washington, D.C. USA) W. LEE "Human Retinoblastoma Susceptibility Gene: Cloning, Identification and Sequence" entire article.	1,4,8-16
P,X	EP, 0,259031 (Massachusetts Eye and Ear Infirmary) 09 March 1988	1,4,8-16
Y	Chemical Abstracts, Volume 107, No. 9 issued August 1987 (Columbus, Ohio, USA) S.A. LATT "DNA based detection of chromosome deletion & amplification" abstract No. 71579;, Cold Spring Harbor Symp. Quant. Biol. 51(1) 299-307	1,4,8-16
X Y	Chemical Abstracts, Volume 106 No. 7, issued February 1987 (Columbus, Ohio, USA) H. Scheffer, "A straightforward approach to isolate DNA sequences with potential linkage to the retinoblastoma locus" abstract No. 44946Z, Hum. Genet. 74(3) 249-255	14 8-13,15,16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 April 1989	08 JUN 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	SCOTT A. CHAMBERS

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Chemical Abstracts, Volume 105, No.17, issued October 1986 (Columbus, Ohio, USA) E.Y. LEE, "Molecular cloning of the human esterase D gene a genetic marker of retinoblastoma" abstract No. 147520q, Proc. Natl. Acad. Sci. U.S.A. 83(17) 6337-41.	14

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1, 4 and 8-16
II. Claims 2 and 3
III. Claims 5, 17 and 18
IV. Claims 6, 7 and 19-21

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1, 4 and 8-16

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Chemical Abstracts, Volume 102, No. 7 issued February 1985 (Columbus, Ohio, U.S.A.) M. LALANDE "Isolation of human chromosome 13-specific DNA sequences cloned from flow sorted chromosome and potentially linked to the retinoblastoma locus" abstract No. 57143C, Cancer Genet. Cytogenet 13(4) 283-95.	1,4,8-16